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"MiRNA expression in Primary Myelofibrosis: characterization and pathophysiology implications"

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INDEX

1 ABSTRACT

Primary myelofibrosis (PMF) is a myeloproliferative neoplasm characterized by megakaryocyte (MK) hyperplasia, bone marrow fibrosis, and abnormal stem cell trafficking. PMF may be associated with somatic mutations, about 90% of patients harbor one of three "driver" mutations, with mutational frequencies of approximately 60%, 22% and 6% for JAK2, CALR and MPL, respectively. Other "non-driver" mutations have also been described in PMF involving different cellular targets such as epigenetic regulatory pathways genes (*ASXL1, DNMT3A, EZH2, IDH1 and IDH2, TET2*), splicing factor genes (SRSF2, SF3B1) and progression to leukemia (CBL, LNK, RUNX1, TP53). However, several aspects of its pathogenesis remain elusive. Increasing evidences indicate that the deregulation of microRNAs (miRNAs) might plays an important role in hematologic malignancies, including MPN. In this work, in collaboration with a group at the *Hemopoietic Stem Cells Laboratory* of the University of Modena and Reggio Emilia and a bioinformatics group of University of Padua, it was performed a genome wide analysis of coding RNA (GEP) and microRNA (miEP) expression in $CD34^+$ cells purified from patients with PMF and from health subjects initially using Affymetrix technology. By means of miRNA-gene expression integrative analysis, it was found different regulatory networks involved in the dysregulation of transcriptional control and chromatin remodeling. In particular, it was identified a network gathering several miRNAs with oncogenic potential (e.g. miR-155-5p) and targeted genes whose abnormal function has been previously associated with myeloid neoplasms, including *JARID2, NR4A3, CDC42*, and *HMGB3.* Because the validation of miRNA-target interactions unveiled *JARID2/miR-155-5p* as the strongest relationship in the network, it was studied the function of this axis in normal and PMF $CD34^+$ cells. This study showed that *JARID2* downregulation mediated by miR-155-5p overexpression leads to increased in vitro formation of $CD41^+$ MK precursors. These findings suggest that overexpression of miR-155-5p and the resulting downregulation of *JARID2* may contribute to MK hyperplasia in PMF.

At the same time, to attain deeper and more extensive knowledge of short RNAs (sRNAs) expression pattern in $CD34^+$ cells and of their possible role in mediating post-transcriptional regulation in PMF, $CD34^+$ cells from healthy subjects and PMF patients were sequenced with Illumina HiSeq2000 technology. It was detected the expression of 784 known miRNAs, with a prevalence of miRNA up-regulation in PMF samples, and discovered 34 new miRNAs and 99 new miRNA-offset RNAs (moRNAs) in $CD34^+$ cells. Thirty-seven small RNAs were differentially expressed in PMF patients compared with healthy subjects, according to microRNA sequencing data. Five miRNAs (miR-10b-5p, miR-19b-3p, miR-29a-3p, miR-379-5p, and miR-543) were deregulated also in PMF granulocytes. Moreover, 3'-moR-128-2 resulted consistently downregulated in PMF according to RNA-seq and qRT-PCR data both in $CD34^+$ cells and granulocytes. Target predictions of these validated small RNAs de-regulated in PMF and functional enrichment analyses highlighted many interesting pathways involved in tumor development and progression, such as signaling by *FGFR* and *DAP12* and Oncogene

Induced Senescence. As a whole, data obtained in this study deepened the knowledge of miRNAs and moRNAs altered expression in PMF CD34$^+$ cells and allowed to identify and validate a specific small RNA profile that distinguishes PMF granulocytes from those of normal subjects. It was thus provided new information regarding the possible role of miRNAs and, specifically, of new moRNAs in this disease.

The expression of 175 miRnas in plasma samples was also analyzed in the patients with PMF and the healthy donors and it was identified the presence of 6 differentially expressed miRNAs deregulated in significant statistically way (P value <0.05): miR-let7b*, miR-10b-5p, miR-424 and miR-99a were resulted up-regulated instead miR-144* and miR-375 were down-regulated in PMF patients. These data show a distinct plasma miRNA expression patterns in patients with PMF compared with health subjects which could have a potential utility as prognostic biomarkers. Finally, in order to clarify the contribution of microRNAs also in to the pathogenesis of *JAK2V617F*-positive MPNs, it was analysed the miRNAs expression pattern in erythroid (TER119$^+$) and mieloid (GR1$^+$) cells purified from BM of *JAK2V617F* knock-in (KI) mouse model using TaqMan® Real time PCR. In this part of the study, it was identified a list of differentially expressed miRNAs also in *JAK2V617F* KI mouse whose deregulation might contribute to the development and phenotype of MPNs.

The results of this work provided novel data regarding the expression profile of small RNA expressed in CD34$^+$, granulocytes and plasma of PMF patients; in addition, for the first time a new moRNAs was described as possible contributors to disease pathogenesis. Finally, it was identified a list of differentially expressed miRNAs in *JAK2V617F* KI mouse whose deregulation might contribute to the development/phenotype of MPNs. This information may represent the basis for further studies aimed at a deeper knowledge of the prognostic and therapeutical role of miRNAs and also moRNAs in PMF.

2 INTRODUCTION

2.1 MYELOPROLIFERATIVE NEOPLASMS

The Myeloproliferative disorders comprise several clonal hematologic diseases that are thought to arise from a transformation in a hematopoietic stem cell.

The main clinical features of these diseases are the overproduction of mature, functional blood cells and a long clinical course (Campbell et al., 2006).

William Dameshek was the first to introduce the term "Myeloproliferative disorders (MPD)" to encompass Chronic Myelogenous leukemia (CML), Polycythemia Vera (PV), Essential Thrombocythemia (ET), and Primary Myelofibrosis (PMF) and he called attention to the clinical and bone marrow morphologic similarities between these diseases (Tefferi et al., 2008).

Dameshek's almost 60-year-old insight regarding the pathogenesis of MPD proved to be accurate in that all four MPD originate from a common ancestral clone (or oligoclones) that arises from a polyclonal, but disease susceptible, stem cell pool. Considering this current understanding of the clonal structure in MPD and their propensity to transform in to acute myeloid leukemia (AML), it was appropriate for the 2008 WHO classification system subcommittee to recommend change in terminology from MPD to "Myeloproliferative Neoplasms" (MPN).

The 2008 WHO MPN category includes not only CML, PV, ET, and PMF, defined "Classic" MPN , but also "non classic" MPN group, wich include chronic neutrophilic leukemia (CNL), chronic eosinophilic leukemia-not otherwise specified (CEL-NOS), systemic mastocytosis (SM), and MPN unclassifiable. The MPNs were in turn considered as one of four major categories of chronic myeloid neoplasms, the other three being myelodysplastic syndromes (MDSs), MDS/MPN and "Myeloid and lymphoid neoplasms associed with eosinophilia and abnormalities of *PDGFRA, PDGFRB or FGFR1*" **(**See **Table 1)** (Tefferi et al., 2015).

In the group fo "Classic" MPNs, CML is a myeloproliferative disorder that characterized by the translocation between chromosomes 9 and 22 [t(9;22) (q34;q11.2)], which resulting in the formation of the Philadelphia (Ph)-chromosome containing the BCR-ABL 1 fusion gene, the molecular landmark of 100% of cases of this disease. Therefore, "Classic" MPNs have been further functionally classified based on the presence or absence of this t(9;22) chromosomal translocation in the Philadelphia (Ph) and it's now well established that the three main classical Ph-negative MPNs are PV, ET and PMF.

In the MPNs and the other chronic myeloid neoplasms are characterized by recurrent somatic mutations in different genes the frequency of which is shown in **Table 2**. A detailed discussion of these mutations will be made later.

1. Acute myeloid leukemia (AML) and related precursor neoplasms[a]
2. Myeloproliferative neoplasms (MPN)
2.1. Classic MPN
2.1.1. Chronic myelogenous leukemia, *BCR/ABL1*-positive (CML)
2.1.2. Polycythemia vera (PV)
2.1.3. Primary myelofibrosis (PMF)
2.1.4. Essential thrombocythemia (ET)
2.2. Nonclassic MPN
2.2.1. Chronic neutrophilic leukemia (CNL)
2.2.2. Chronic eosinophilic leukemia, not otherwise specified (CEL-NOS)
2.2.3. Mastocytosis
2.2.4. Myeloproliferative neoplasm, unclassifiable (MPN-U)
3. Myelodysplastic syndromes (MDS)
3.1. Refractory cytopenia[b] with unilineage dysplasia (RCUD)
3.1.1. Refractory anemia (ring sideroblasts < 15% of erythroid precursors)
3.1.2. Refractory neutropenia
3.1.3. Refractory thrombocytopenia
3.2. Refractory anemia with ring sideroblasts (RARS; dysplasia limited to erythroid lineage and ring sidero-blasts [3] 15% of bone marrow erythroid precursors)
3.3. Refractory cytopenia with multilineage dysplasia (RCMD; ring sideroblast count does not matter)
3.4. Refractory anemia with excess blasts (RAEB)
3.4.1. RAEB-1 (2%–4% circulating *or* 5%–9% marrow blasts)
3.4.2. RAEB-2 (5%–19% circulating *or* 10%–19% marrow blasts or Auer rods present)
3.5. MDS associated with isolated del(5q)
3.6. MDS, unclassifiable
4. MDS/MPN
4.1. Chronic myelomonocytic leukemia (CMML)
4.2. Atypical chronic myeloid leukemia, *BCR/ABL1*-negative
4.3. Juvenile myelomonocytic leukemia (JMML)
4.4. MDS/MPN, unclassifiable
4.4.1. Provisional entity: Refractory anemia with ring sideroblasts associated with marked thrombocytosis (RARS-T)
5. Myeloid and lymphoid neoplasms with eosinophilia and abnormalities of *PDGFRA*,[c] *PDGFRB*,[c] or *FGFR1*
5.1. Myeloid and lymphoid neoplasms with *PDGFRA* rearrangement
5.2. Myeloid neoplasms with *PDGFRB* rearrangement
5.3. Myeloid and lymphoid neoplasms with *FGFR1* abnormalities

[a]Acute myeloid leukemia-related precursor neoplasms include "therapy-related myelodysplastic syndrome" and "myeloid sarcoma".
[b]Either mono- or bicytopenia: hemoglobin level < 10 g/dL, absolute neutrophil count < 1.8×10^9/L, or platelet count < 100×10^9/L. However, higher blood counts do not exclude the diagnosis in the presence of unequivocal histological/cytogenetic evidence for myelodysplastic syndrome.
[c]Genetic rearrangements involving platelet-derived growth factor receptor a/b (*PDGFRA/PDGFRB*) or fibroblast growth factor receptor 1 (*FGFR1*).

Tab. 1: The 2008 World Health Organization Classification for Myeloid Neoplasms.

Disease Associated mutation(s) (estimated frequency)	Myelodysplastic syndrome *TET2* (20%) Refractory anemia with ring sideroblasts *SF3B1* (80%-90%)	Chronic myelomonocytic leukemia *TET2* (40%-60%) *SRSF2* (30%-50%) *ASXL1* (40%) Refractory anemia with ring sideroblasts associated with marked thrombocytosis *SF3B1* (80%-90%) *JAK2*V617F (50%) Atypical chronic myeloid leukemia *SETBP1* (30%) Myelodysplastic/myeloproliferative neoplasm–unclassifiable	Chronic myeloid leukemia *BCR-ABL1* (100%) Polycythemia vera *JAK2* (99%) Essential thrombocythemia *JAK2/CALR/MPL* (85%) Primary myelofibrosis *JAK2/CALR/MPL* (90%) Chronic neutrophilic leukemia *CSF3R* (80%-100%) *SETBP1* (30%) Systemic mastocytosis *KIT*D816V (80%-100%) Chronic eosinophilic leukemia Myeloproliferative neoplasm–unclassifiable	Myeloid and lymphoid neoplasms with eosinophilia and *PDGFRA*, *PDGFRB*, or *FGFR1* mutations

Tab. 2: Chronic Myeloid Neoplasm and relative associated mutation (Tefferi et al., 2015).

2.2 THE "CLASSIC" Ph-NEGATIVE MYELOPROLIFERATIVE NEOPLASM.

This study is focused on the three main Classical Ph-negative MPN and, in particular on the PMF disease. The classification is based on which myeloid cell lineage is predominantly expanded in the blood; in PV and ET there are respectively elevated red cell mass and elevated platelet number. PMF is more complex disease and patients display bone marrow fibrosis, variable myeloid cell number, extramedullary hematopoiesis and hepatosplenomegaly.
Patients with PV and ET have a high risk of thrombotic and/or haemorrhagic events and may progress to an accelerated myelofibrosis phase, while all three subtypes are associated with a long term risk of transformation to acute myeloid leukemia (AML) with a uniformly poor prognosis. The risk of leukemic transformation is highest in PMF, where it is estimated to be approximately 10-20% at 10 years. In PV the risk is 2.3 % at 10 years and 7.9 % at 20 years (Tefferi et al., 2014). Transformation to AML is considered relatively uncommon in ET (Vardiman et al., 2009).

2.2.1 POLYCYTHEMIA VERA AND ESSENTIAL THROMBOCYTHEMIA.

PV is a pathologic condition presenting an increase of red blood cells as a result of the autonomous and clonal proliferation of bone marrow stem cells **(Fig.1)**.
Described for the first time by Vaquez (Vaquez et al., 1892) in 1892 and singled out as a clinical entity by Osler (Osler et al., 2008), it was considered a slow but fatal disease up to a few years ago. However, in the last decade, changes in the physiopathologic criteria and the identification of the *JAK2* protein mutation (*JAK2V617F*) led the way to diagnostic advances, better risk classification and the development of new therapies.

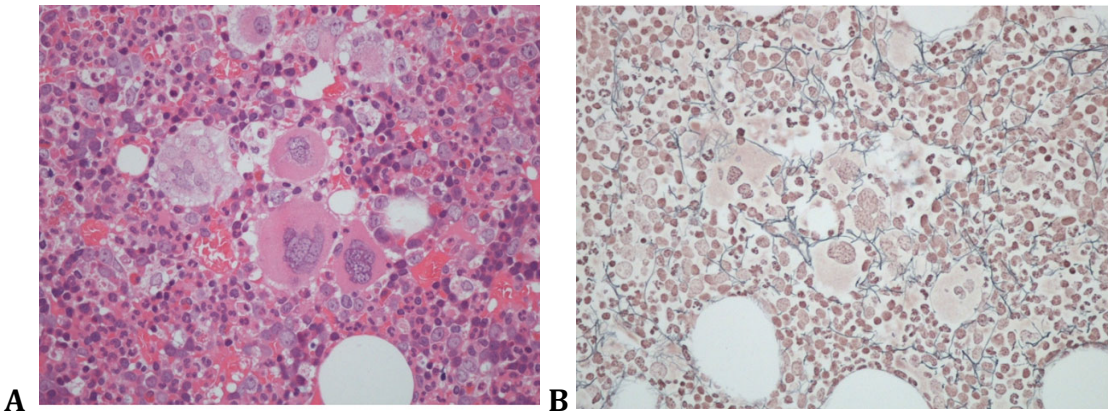


Fig. 1: (A) Typical polycythaemia vera histology with panmyelosis and pleomorphic, clustered megakayrocytes. Haematoxylin and Eosin (H&E) stain; original magnification 940. **(B)** Borderline increase in reticulin fibres (World Health Organization fibrosis score 0–1) accompanying panmyelosis. Gomori silver stain for reticulin; original magnification 940..

The annual incidence of PV is ~2 per 100,000 inhabitants, respectively, if based on large population surveys and adjusted to a standard population.

While there is no known familial disposition to PV, rare families have been described in which multiple members develop myeloproliferative neoplasms, including PV (Bellanné-Chantelot C. et al., 2006). Studies of these families suggest the presence of an autosomal dominant mutation that may predispose to acquisition of a secondary somatic mutation such as a *JAK2* mutation.

	Polycythemia vera
Major criteria	1. Hgb > 18.5 g/dL (men), Hgb > 16.5 g/dL (women) or Hgb > 17 g/dL (men), Hbg > 15 g/dL (women) if associated with an increase of ≥ 2 g/dL from baseline that cannot be attributed to correction of iron deficiency 2. Hgb or Htc > 99th percentile of reference range for age, gender or altitude of residence or red cell mass elevated > 25% above mean normal
Minor criteria	1. Presence of JAK2V617F mutation bone marrow trilineage myeloproliferation 2. Subnormal serum Epo level 3. EEC growth.

Adapted from Tefferi & Vardiman, 2008 [6]
EEC - endogenous erythroid colony; Epo - erythropoietin; Htc - hematocrit; Hgb - hemoglobin
Diagnosis of polycythemia vera requires either both major criteria and one minor criterion or the first major criterion and 2 minor criteria

Tab. 3: WHO Diagnostic Criteria for Polycythemia Vera

Signs and symptoms of PV are caused by a higher erythrocytic load, leading to increases in blood viscosity and as a consequence to the decrease of its flow. It is seen in middle age individuals who characteristically present a cyanotic blush, more easily seen in the face, mainly lips, nose tip and ears. The same color alteration is also found in the distal portion of the limbs (Bellanné-Chantelot C. et al., 2006). Increased blood pressure is frequently found in PV and this disease may be the cause of imbalance in the arterial hypertension. Common complaints are headaches, tinnitus, paresthesia and erythromelalgia which are related to the vaso occlusive phenomena (Bellanné-Chantelot C. et al., 2006). Pruritus may occur after a hot or tepid baths and there can be some exacerbation of gout. All of these symptoms

tend to improve or disappear with treatment. The majority of patients present splenomegaly of varying degrees at diagnosis or during the evolution of the disease. They complain about feeling early satiety, increased abdominal volume, left hypochondriac pain or bowel constipation (Bittencourt et al., 2012).

The diagnosis is based on the British guidelines and on the WHO 2008 criteria and requires the integration of clinical, laboratorial, and histology factors **(Table 3)**. These are important points for proving the hematic changes by measurements of erythrocytic mass and Hb, as well as demonstrating the presence of *JAK2V617F* or other mutations having a similar function.

The bone marrow is hypercellular in all sectors, mainly in the erythrocytic and megakaryocytic lineages. Erythroid and myeloid precursors are morphologically normal whereas megakaryocytes may present in loose and hyperlobulated groups located close to the bone trabecula. The requirement is the presence of both major criteria and at least one of the minor ones, or the first major criterion associated to two minor criteria.

ET is characterized by megakaryocytic proliferation with consequent increased peripheral platelets **(Fig 2)**. Its course is chronic and may progress to myelofibrosis or acute leukemia (Tefferi et al., 2008)**.**

The incidence varies between 0.6 to 2.5 cases per 100,000 persons/year. (Johansson, 2006). It is a disorder of middle age and is rare in childhood (one case per one million children). It is predominant in females (2:1). It is often diagnosed following incidental finding of a high platelet count, although some patients present with thrombotic or haemorrhagic complication. There is possibly family involvement.

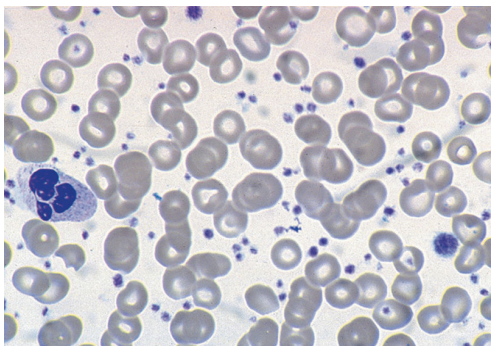
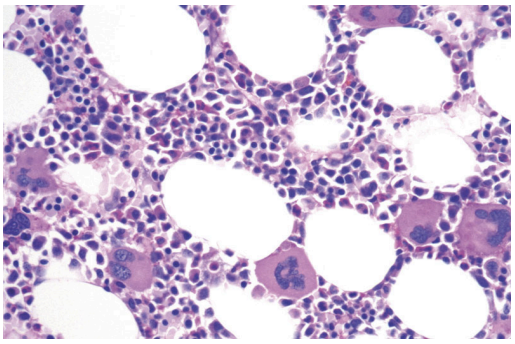
A  **B** 

Fig.2: Essential thrombocythaemia, **A**: Peripheral blood smear. The major abnormality seen is marked thrombocytosis. The platelets show anisocytosis, but are often not remarkably atypical;
B: Bone marrow biopsy. The enlarged megakaryocytes (PAS staining). They reveal abundant amounts of mature cytoplasm and deeply lobulated and hyperlobulated (stag-horn-like) nuclei.

ET is an indolent disorder and affected patients have a long survival. The main risk is thrombosis, while myelofibrosis and leukemia are rare and late complications.

This disease was attributed to the proliferative advantage of megakaryocytic factors related to thrombopoietin (TPO) and its cellular receptor (c-Mpl). The mutations involved in MPN start early in the stem cell. Megakaryocytic precursors are hypersensitive to TPO and the action of cytokines: TGF beta, Interleukins 3 (IL-3), IL-6 and IL-11, which interact with the bone marrow microenvironment, promoting the

growth of megakaryocytes. In 35 to 70% of cases, acquired somatic mutation occur in the Janus kinase 2 (*JAK2V617F*), responsible for phosphorylation and signaling activators of transcription (STAT). Three novel somatic mutations in the juxtamembrane region at codon 515 *MPL* (W515L, W515K, S505N) explain defects in signaling pathways of molecules STAT3, STAT5, mitogen activator protein (MAPK) and phosphatidylinositol-3kinase AKT (PI3K/AKT), giving a gain in function and proliferative advantage to megakaryocytes (Girodon et al., 2009).

In the WHO guidelines published in 2008 the diagnostis criteria are four **(Table 4)**.

The platelet threshold is lowered to $450x10^9$/L, as the earlier use of a threshold level of 600 x 10^9/L compromises the detection of early-phase disease, since the 95th percentile for normal platelet count, adjusted for gender and race, is below 400 x 10^9/L. The demonstration of *JAK2V617F* mutation that is present in 50-55% of ET patients, or the demonstration of other clonal marker, is included. But since the importance of the *JAK2V617F* marker is limited by its lower frequency in ET, a bone marrow biopsy is still required (as in the former 2001 WHO criteria) (Thiele et al., 2006) to help with the differential diagnosis between *JAK2V617F*-negative ET and reactive thrombocytosis and to differentiate ET from other chronic myeloid neoplasms, including cellular phase/prefibrotic PMF and myelodysplastic syndromes (MDS). Of special consideration is the differentiation between ET and PMF grade 0 (CPMF0), which shows a different morphology of the megakaryocytes (smaller, hypolobulated and clustered).

1. Sustained[a] platelet count ≥450x10^9/L
2. Bone marrow biopsy specimen showing proliferation mainly of the megakaryocytic lineage with increased numbers of enlarged, mature megakaryocytes. No significant increase or left-shift of neutrophil granulopoiesis or erythropoiesis
3. Not meeting WHO criteria for polycythaemia vera,[b] primary myelofibrosis,[c] *BCR-ABL1* positive chronic myelogenous leukaemia[d] or myelodysplastic syndrome[e] or other myeloid neoplasm
4. Demonstration of *JAK2* V617F or other clonal marker, or in the absence of *JAK2* V617F, no evidence for reactive thrombocytosis[f]

[a] Sustained during the work-up process.
[b] Requires the failure of iron replacement therapy to increase haemoglobin level to the polycythaemia vera range in the presence of decreased serum ferritin. Exclusion of polycythaemia vera is based on haemoglobin and haematocrit levels and red cell mass measurement is not required.
[c] Requires the absence of relevant reticulin fibrosis, collagen fibrosis, peripheral blood leukoerythroblastosis, or markedly hypercellular marrow accompanied by megakaryocyte morphology that is typical for primary myelofibrosis including small to large megakaryocytes with an aberrant nuclear/cytoplasmic ratio and hyperchromatic, bulbous or irregularly folded nuclei and dense clustering.
[d] Requires the absence of *BCR-ABL1*.
[e] Requires absence of dyserythropoiesis and dysgranulopoiesis.
[f] Causes of reactive thrombocytosis include iron deficiency, splenectomy, surgery, infection, inflammation, connective tissue disease, metastatic cancer, and lymphoproliferative disorders. However, the presence of a condition associated with reactive thrombocytosis may not exclude the possibility of ET if the first three criteria are met.

Tab. 4: WHO Diagnostic Criteria for Essential Thrombocythemia.

An important addition to our knowledge has been the discovery of new mutations in calreticulin (*CALR*) gene in 2013 (Nangalia et al., 2013; Klampfl et al., 2013). These alterations account for approximately 30% of cases of essential thrombocythemia and with the *JAK2V617F* mutation, the thrombopoietin receptor *MPL* W515K/L mutation are mutually exclusive in essential thrombocythemia and support a novel molecular categorization of essential thrombocythemia.

After the first clinical approach in according to WHO criteria, when evaluating

thrombocytosis, the detection of *JAK2V617F, MPL* and also *CALR* mutations confirms the presence of an underlying MPN but their absence does not exclude the possibility since up to 20% of patients with ET might be triple negative. In these cases the bone marrow morphology is crucial for the diagnosis (Tefferi and Barbui, 2015).

2.2.2 MYELOFIBROSIS

The term myelofibrosis (MF) refers to primary MF (PMF) (Tefferi et al., 2007) and to the phenotypically overlapping conditions that develop as (usually) late evolution of either polycythemia vera (PPV-MF) or essential thrombocythemia (PET-MF) (Barosi et al., 2008). Most information available in the literature concerns PMF, and rigorously defined criteria for risk stratification have been developed specifically for PMF.

These disease occurs in 0.5- 1.5 cases per 100,000 people per year, most commonly in the sixth or seventh decades of life and affecting both genders. Rarely, MF can occur from the neonatal period to the ninth decade of life. In infants the disorder can mimics the classic disease or show certain features but not others, such as absence of hepatosplenomegaly (Sekhar et al., 1996).

Among the MPN Ph negative, MF is the worst among the chronic myeloproliferative neoplasms in terms of survival (4-5.5 years) and quality of life (Cervantes et al, 2012). In addition, when the survival of patients with this disease was compared with that of individuals of the same age, and the same gender in the general population, it saw a 31% reduction in life expectancy (Barbui et al., 2012). Even the survival of patients with myelofibrosis secondary does not seem to differ from those with PMF.

The main causes of death are: evolution in acute leukemia (in 20% of cases at 10 years after diagnosis), infection and bleeding secondary to bone marrow failure, portal hypertension or liver failure secondary to thrombosis of the hepatic veins, venous thrombosis or metaplasia myeloid liver, thrombosis in other territories and heart failure (Barbui et al., 2012).

25% of patients with MF is completely asymptomatic and generally turns out to have the disease after more extensive investigations made following a simple analysis of blood or a peripheral blood smear in which abnormalities are detected, or again, following a ultrasound done for different reasons in which it discovers the presence of an enlarged spleen (splenomegaly is a typical characteristic of patients with MF).

On the other hand, in patients with symptoms, the most common factor is the fatigue caused by anemia, a typical feature of the disease and greatly affects the quality of life of these patients. Other nonspecific symptoms include fever, night sweats, itching, bone pain and weight loss that are present at diagnosis in 20-50% of patients, to a greater extent in the elderly. In addition it can also occur early satiety and gastric emptying slow as consequences of the pressure on the stomach from the spleen (Barbui et al., 2012).

- Pathogenesis

PMF is characterized in the WHO 2008 classification (major criterion) as: "presence of megakaryocyte proliferation and atypia, usually accompanied by reticulin and/or collagen fibrosis, or in the absence of significant reticulin fibrosis, the megakaryocytic changes must be accompanied by an increased bone marrow cellularity characterized by granulocytic proliferation and often decreased erythropoiesis (es.: prefibrotic cellular-phase disease).

There may be a moderate increase in reticulin fibers, but no collagen is seen. Megakaryocyte alterations are rather discriminating: anomalous topography, with prominent morphological abnormalities: pleomorphism, aberration of the nuclear cytoplasmic ratio due to large, bulbous and hyperchromatic cloud-shaped nuclei, disorganization of nuclear lobulation, naked megakaryocytes and maturation arrest. Early fibrosis leads to sinus dilatation as a result of stromal retraction (Ahmed et al., 2006) **(Fig.3)** .

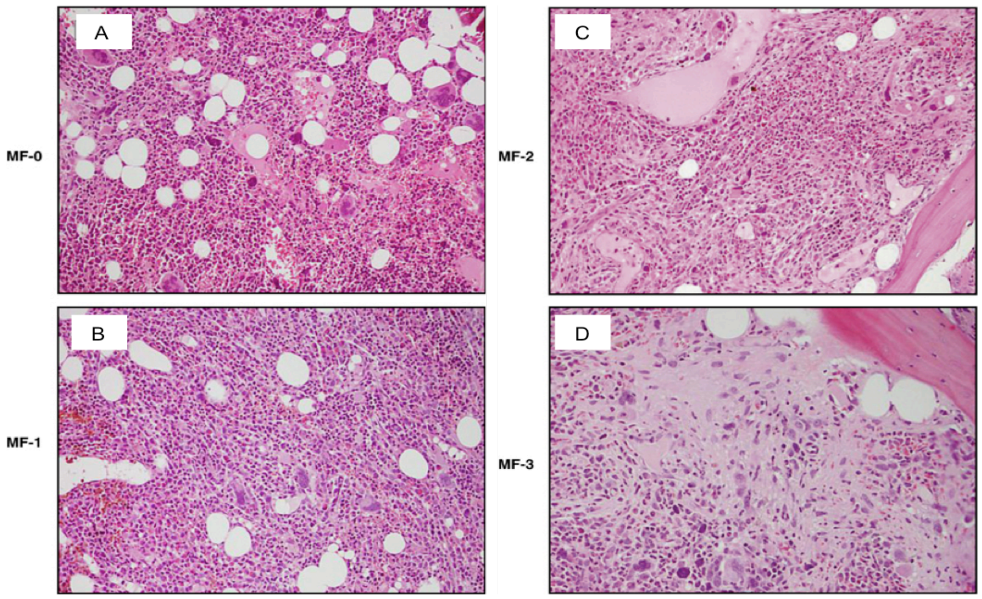


Fig.3: Example of morphological feature of primary myelofibrosis and grading of bone marrow fibrosis. (A) Pre-fibrotic primary myelofibrosis (MF-0) ; (B) Early-stage primary myelofibrosis (MF-1): loose network of reticulin fibres with many intersection, especially in perivascular areas. (C) Fibrotic stage primary myelofibrosis (MF-2) : diffuse and dense increase in reticulin fibres, with extensive intersection and occasionally with focal bundles of collagen. (D) Fibrotic stage primary myelofibrosis (MF-3): diffuse and dense increase in reticulin fibres, with extensive intersections and coarse bundles of collagen.

Studies by Vannucchi A. et al have shown the central role of megakaryocytes in the pathogenesis of the disease and the importance of fibrogenic cytokines such as the Transforming Grow factor-beta (TGF-β) in the production of collagen and reticulin go to constitute the fibers in the bone (Vannucchi, Bianchi et al., 2005).

TGF-β is the most important growth factor in the biogenesis of marrow fibrosis. Its main functions are:

• stimulation of fibroblasts to the medullary production of pro collagen type I and III and to the synthesis of fibronectin;

• inhibition of enzymes responsible for the degradation of the extracellular matrix collagenase-like;
• induction to the synthesis of inhibitors of proteases such as plasminogen activator inhibitor. The resulting picture is the accumulation of extracellular matrix which contributes to a further progression of fibrosis. The MF, therefore, could be linked, on the one hand, the increase in the number of megakaryocytes and their maturation is not complete and that, consequently, the release of high amounts of growth factors present in the granules α (such as the TGF-β) (Chagraoui, Komura et al., 2002; Vannucchi, Bianchi et al., 2005).
To confirm the fact that the histological changes of the bone marrow are mediated by cytokines has been seen that both the cellular levels that extracellular levels of fibrogenic cytokines and angiogenic properties are altered in patients with myelofibrosis (Ramashwar et al., 1994). In the MF, and then, the bone marrow fibrosis occurs in response to a clonal proliferation of hematopoietic stem cells that lead to a profound morphologically abnormal hyperplasia of megakaryocytes and populations of monocytes which release growth factors fibrogenic. What follows from the clinical point of view, finally, it was a result of the alteration of the hematopoietic stem cell and its progeny rather than related to the event of simple marrow fibrosis (Hofmann et al., 2007). The second factor, neoangiogenesis, in particular in bone marrow, due to the proliferation of endothelial cells has been postulated to be mediated by an increase in the production and release of factors such as b-FGF and VEGF by megakaryocytes. The involvement of the latter suggests a possible link between angiogenesis and fibrosclerosis medullary (Barbui, 2012). Finally, in addition to these two characteristics, considerable importance is the amount of $CD34^+$ stem cells that are a undifferentiated progenitor cells of the myeloid lineage, present in individuals with MF about 360 times greater not only compared to normal controls, but also with respect to patients with TE or PV, from 18 to 30 times more. The number of circulating $CD34^+$ cells tends to increase with the progression of the disease and there is a close correlation between a very large number (> $300x10^6$ / L) and the upcoming leukemic evolution (Barosi et al., 2012). Compared to controls, in patients with MF they are present in the circulation also many more pluripotent cells, in particular, the colony-forming units CFU-MK (colony-forming unit-megakaryocyte), and also CFU-GM (colony forming unit granulocyte-monocyte), BFU-E (burst-forming unit erythrocyte), CFU-GEMM (colony forming unit granulocyte, erythroid, monocyte, megakaryocyte).
About animal models, transgenic and knock-in mice expressing mutant *JAK2* have provided compelling evidence that mutated *JAK2* (typically *JAK2V617F*) is a driver in this major subset of myeloproliferative neoplasms, however these mice are poor models for PMF. PMF characteristics such as megakaryocyte proliferation and fibrosis have been recapitulated in mice expressing thrombopoietin (Villeval et al., 1997), the NF-E2 transcription factor (Kaufmann et al., 2012), vascular endothelial growth factor (Maes et al., 2010) or reduced levels of GATA1 (Vannucchi et al., 2008), suggesting that abnormal erythroid/megakaryocyte development and/or abnormal release of cytokines may be a key factor in the disease.

- Diagnosis of PMF

Current diagnosis of PMF is based on the 2008 World Health Organization (WHO) criteria, (Tefferi et al., 2008) (which enlist histopathologic, morphologic, clinical, and molecular-cytogenetic variables). Histopathology is key to the diagnosis, demonstrating the atypia of megakaryocyte proliferation even in the absence of overt reticulin fibrosis; it also helps in the differential diagnosis with nonclassical MPN disorders, myelodysplastic syndromes including the del(5q) syndrome, PV or ET with initial BM fibrosis, and other myeloid neoplasms. An other major diagnostic criterion is represented by the detection of *JAK2*V617F or a *MPL* mutation, occurring in 60% and 5%-10% of the patients, respectively, and other MPN-associated molecular abnormalities (eg, *CBL, ASXL1, TET2*, and *EZH2*) or clonal markers (particularly trisomy 9 or 13q−) that distinguish PMF from reactive marrow fibrosis (Tefferi et al, 2010) absence of BCR/ABL rearrangement allows the exclusion of chronic myelogenous leukemia. Minor criteria are represented by leukoerythroblastosis, raised serum lactate dehydrogenase levels, anemia, and splenomegaly **(See Table 5).**

Major criteria

1. Presence of megakaryocyte proliferation and atypia[a], usually accompanied by either reticulin and/or collagen fibrosis, or
   in the absence of significant reticulin fibrosis, the megakaryocyte changes must be accompanied by an increased bone marrow cellularity characterized by granulocytic proliferation and often decreased erythropoiesis (i.e. prefibrotic cellular-phase disease).
2. Not meeting WHO criteria for polycythaemia vera[b], *BCR-ABL1*+ chronic myelogenous leukaemia[c], myelodysplastic syndrome[d], or other myeloid neoplasms
3. Demonstration of *JAK2* V617F or other clonal marker (e.g. *MPL* W515K/L), or
   in the absence of a clonal marker, no evidence that the bone marrow fibrosis or other changes are secondary to infection, autoimmune disorder or other chronic inflammatory condition, hairy cell leukaemia or other lymphoid neoplasm, metastatic malignancy, or toxic (chronic) myelopathies[e]

Minor criteria

1. Leukoerythroblastosis[f]
2. Increase in serum lactate dehydrogenase level[f]
3. Anaemia[f]
4. Splenomegaly[f]

[a] Small to large megakaryocytes with an aberrant nuclear/cytoplasmic ratio and hyperchromatic, bulbous, or irregularly folded nuclei and dense clustering.
[b] Requires the failure of iron replacement therapy to increase haemoglobin level to the polycythaemia vera range in the presence of decreased serum ferritin. Exclusion of polycythaemia vera is based on haemoglobin and haematocrit levels, and red cell mass measurement is not required.
[c] Requires the absence of *BCR-ABL1*.
[d] Requires absence of dyserythropoiesis and dysgranulopoiesis.
[e] Patients with conditions associated with reactive myelofibrosis are not immune to PMF, and the diagnosis should be considered in such cases if other criteria are met.
[f] Degree of abnormality could be borderline or marked.

Tab. 5: WHO Diagnostic Criteria for Primary Myelofibrosis.

Despite the diagnostic criteria used in the different classifications are reproducible and clinically useful, the current WHO classification does not take into account some aspects molecular recently identified in MPN, without ensuring complete diagnostic specificity. With the discovery of mutations in the gene *CALR* (which will be discussed in more detail later), the 90% of patients with ET and PMF are characterized by at least one alteration clonal specific enough to confirm the diagnosis of MPN. In light of this, in a recent paper by Tefferi et al., It has proposed a revision of the 2008 WHO diagnostic criteria determining what is reasonable to expect to make the diagnosis of PMF in the presence of three main criteria such as: (1) a typical morphology in the bone marrow, (2) the absence of evidence for another myeloid malignancy who CML, PV or ET, and (3) the presence of mutations in *JAK2, CALR or MPL*. In the absence of mutations in *JAK2*, *CALR* or *MPL* (ie cases called "triple negative"), the diagnosis of PMF would require not only the presence of the first two criteria but also more the exclusion of reactive marrow fibrosis and the presence of clinical and laboratory factors that are typical of the PMF. These additional criteria are now incorporated as minor criteria in the revision scheme that include the presence of anemia and palpable splenomegaly, the presence of leucoeritroblastosis or the increase in lactate

dehydrogenase (LDH) (Tefferi et al, 2014). Once the diagnosis of PMF, in order to decide the best therapeutic strategy to be used for each patient and define the prognosis, should be established in which risk category prognostic belongs.

In fact, just in order to stratify patients into different risk categories, IWG-MRT has initially developed an International Prognostic, the IPSS (International Prognostic Score System) (Cervantes et al, 2009), based on data from Clinical and laboratory features obtained from a series of 1001 patients with PMF. To predict the survival of these patients, this system takes into account five variables, determined at the time of diagnosis: (1) age over 65, (2) hemoglobin levels below 100 g/L, (3) white blood cell count higher to 25 * 10^9 / L, (4) a percentage of blasts in the peripheral blood greater than or equal to 1%, and (5) presence of constitutional symptoms (eg. night sweats, weight reduction of 10% over 6 months, fever devoid of infection).

Based on these parameters, patients were divided into four different classes of risk, the category of low risk, intermediate risk 1, 2 at intermediate risk and high risk. The same parameters used dall'IPSS prognosis have been incorporated in a time-dependent prognostic score, applicable at all times of the natural history of the disease, called DIPSS (Dynamic International Prognostic Scoring System). Each parameter is assigned a single point, with the exception of anemia, which affects more prognosis (2 points). This score can identify patients who have a more aggressive course of the disease, so they can properly orient the therapeutic choices (Passamonti et al, 2010). Other factors not included nell'IPSS who had an impact on survival were represented by the need for transfusions, a platelet count of less than 100 * 10^9 / L and an "unfavorable karyotype defined". These three variables were included in the recently defined a new prognostic score Dynamic International Prognostic Scoring System-plus (DIPSS-plus), which allows a better score prognostic classification **(Table 6)**.

	IPSS [15]	DIPSS [16]	DIPSS-plus [17]
Risk factors	1. Age > 65 = 1 2. Anemia < 10 g/dl = 1 3. WBC > 25 × 10^9/l = 1 4. Blood blasts >1% = 1 5. Constitutional symptoms = 1	1. Age >65 =1 2. Hgb < 10 g/dl =2 3. WBC > 25 × 10^9/l = 1 4. Blood blasts > 1% = 1 5. Constitutional symptoms = 1	1. DIPSS low = 0 2. DIPSS intermediate-1 = 1 3. DIPSS intermediate-2 = 2 4. DIPSS high risk = 3 +1 point each for: 5. Transfusion dependency 6. Unfavorable cytogenetics* 7. Platelets < 100 × 10^9/l
Scoring (risk factors)	Low: 0 Intermediate-1: 1 Intermediate-2: 2 High risk: >3	Low: 0 Intermediate-1: 1–2 Intermediate-2: 3–4 High risk: 5–6	Low: – Intermediate-1: 1 Intermediate-2: 2–3 High risk: 4–6

Tab. 6: Prognostic score System in MF

For each factor is assigned a point , and so, depending on the number of factors present, it is possible to subdivide the patients (also in this case as it did for the IPSS) in four different risk groups : low-risk (0 negative prognostic factors) with median survival of 185 months , intermediate risk-1 (factor 1) with median survival of 78

months , intermediate risk -2 (2 factors) with median survival of 35 months , high risk (at least 3 factors) with median survival of 16 months (Tefferi et al. , 2014) (Fig.4). Since the publication of DIPSS-plus score, several studies that suggest additional prognostic information have been published.

For example Tefferi and colleagues demonstrated that a > 80% 2-year mortality in PMF patients was predicted by monosomal karyotype, inv(3)/i(17q) abnormalities, or any two of circulating blasts >9%, leukocytes ≥ $40*10^9$/L or other unfavourable karyotype (Tefferi et al., 2011).

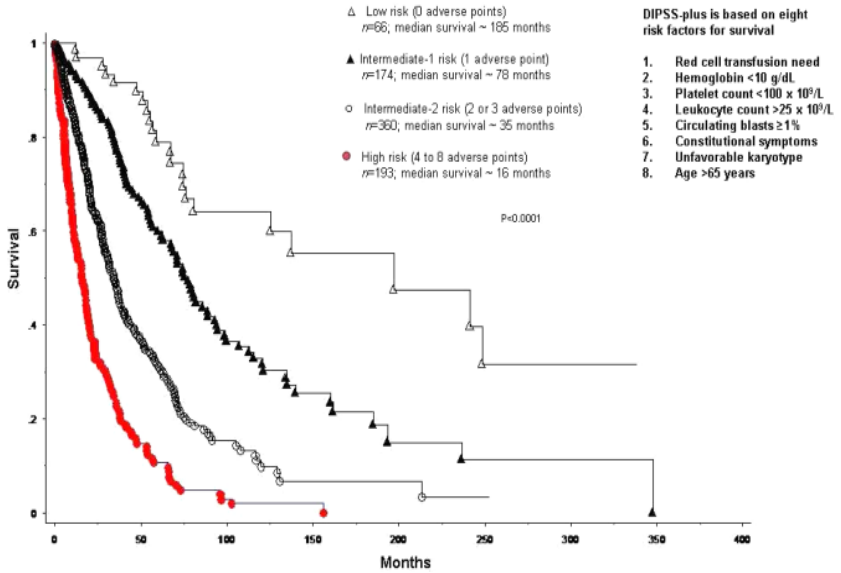


Fig.4: DIPSS-plus (Dynamic international prognostic scoring system 1 karyotype 1 platelet count 1 transfusion status) risk stratification in 793 patients with primary myelofibrosis seen at Mayo Clinic Rochester (with permission from Gangat et al., 2011).

More recently, a number of DIPSS-plus-independent risk factors in PMF were identified and they include "driver mutations" other than type 1 or type 1-like *CALR* variants, (Tefferi et al., 2014) monosomal karyotype, (Vaidja et al., 2011) nullizygosity for *JAK2* 46/1 haplotype, (Tefferi et al., 2010) low *JAK2V617F* allele burden, (Tefferi et al., 2008, Guglielmelli et al., 2009) presence or number (Guglielmelli et al., 2014) of *IDH1/2*, *EZH2*, *SRSF2* or *ASXL1* mutations (Vannucchi et al., 2013) and increased serum IL-8, IL-2R (Tefferi et al., 2011) or serum free light chain levels (Pardanani et al., 2012). In a recent international study of 570 patients with PMF (Pardanai et al., 2012) the authors reported the longest survival in $CALR^+ASXL1^-$ patients (median 10.4 years) and shortest in $CALR^-ASXL1^+$ patients (median 2.3 years). $CALR^+ASXL1^+$ and $CALR^-ASXL1^-$ patients had similar survival and were grouped together in an intermediate risk category (median survival 5.8 years).

Some of the above outlined clinical and genetic risk factors have also been associated with increased risk of leukemic transformation.

- Treatment

Treatment of patients with myelofibrosis is based on symptoms and risk category. Asymptomatic, low risk myelofibrosis (DIPSS-plus 0–1 risk factors) patients may be

observed. Symptomatic, intermediate-2 or high-risk (DIPSS-plus 2–4 risk factors) patients are considered treatment with a JAK2 inhibitor, a clinical trial, or are considered an allogeneic stem cell transplant. Patients that are not candidates for JAK2 inhibitors or stem cell transplant was treated with agents such as androgens, immunomodulatory agents (i.e., thalidomide), interferon, or hydroxyurea (Mesa et al., 2003). Unfortunately, these treatment options are only helpful in a minority of patients, are not usually durable, and do not appreciably alter the biology of the disease (Barbui et al., 2011).

Improved understanding of the disease biology of MPNs has led to the approval of Ruxolitinib, the first oral JAK1/2 inhibitor, by the Food and Drug Administration in 2011 for the treatment of patients with intermediate and high-risk myelofibrosis. The approval of Ruxolitinib was based on two large, international, phase III studies: COMFORT I and II (Verstovsek et al., 2012; Harrison et al., 2012).

The JAK-inhibitors act mainly by inhibiting dysregulated JAK-STAT signalling, present in all MF patients. They are not selective of the mutated JAK2 so they are indicated in both JAK mutated and unmutated MF patients.

In the COMFORT I study, patients with myelofibrosis were randomized to receive Ruxolitinib or placebo **(**Verstovsek et al., 2012). Patients in the COMFORT II study were randomized in a 2 : 1 fashion to receive ruxolitinib or best available therapy. Patients were eligible regardless of their JAK2 mutational status and, importantly, noted to benefit regardless of their JAK2 mutational status. The primary endpoint of both studies was spleen volume reduction of 35% at 24 and 48 weeks for COMFORT I and II, respectively, by MRI. Secondary endpoints in the COMFORT I study included durability of response, improvement in symptom burden as measured by myelofibrosis symptom assessment form, and overall survival (OS). In the COMFORT I study, 42% of patients experienced spleen volume reduction by 24 weeks, and in the COMFORT II study, 28% of patients compared with 0% in the best available therapy group experienced a 35% spleen volume reduction at 48 weeks. In addition, 45% of patients experienced significant symptom improvement in the ruxolitinib group.

The benefits of ruxolitinib were durable and, in addition, both studies reported a significant improvement in OS (results of the COMFORT II study were updated in a recent study with 3-year follow-up confirming an OS benefit). On the basis of these results, patients with intermediate or high-risk myelofibrosis, who have splenomegaly, and/or constitutional symptoms can consider the use of ruxolitinib. It is the first drug approved for MF treatment (Verstovsek et al., 2010).

It should be noted that despite improvement in the understanding of the molecular biology of myelofibrosis and the approval of ruxolitinib, the only curative treatment approach for this disease is allogeneic stem cell transplantation.

By far ASCT (allogeneic stem cell transplantation) is the only treatment modality that can either cure or prolong life and is therefore recommended in either DIPSS-plus high (i.e. presence of at least four of the 8 DIPSS-plus risk factors) (Gargat et al., 2011) or molecularly high (absence of type 1/type 1-like CALR mutation and presence of *ASXL1* or related high risk mutation) (Tefferi et al., 2014) risk disease (Ballen et al.,2010).

Pre-transplant JAK-inhibitor treatment can reduce spleen size and improve constitutional symptoms, but is currently being tested in clinical studies and should be regarded as experimental.

2.3 MOLECULAR BASIS OF MYELOPROLIFERATIVE NEOPLASM

2.3.1.DRIVER MUTATIONS IN MYELOPROLIFERATIVE PH$^-$ DISORDERS.

Our biological understanding of the MPNs was markedly advanced with the discovery of the *JAK2V617F* mutation in 2005 (Levine et al., 2005) . This work and subsequent genetic investigations haverevealed a number of mutations inside and outside of the JAK–STAT pathway. The majority of patients with myelofibrosis have a mutation in *JAK2* , *MPL*, or *CALR* (Nangalia et al., 2013; Klampf et al., 2013) , and these mutations may be thought of as driver mutations in myelofibrosis. Furthermore, all of these mutations appear to exert their effects, at least in part, by activating JAK–STAT target genes.

Indeed, in one analysis of gene expression in MPN patients with *JAK2*, *MPL*, or *CALR* mutations versus controls, gene expression is unable to distinguish between patients with MPN with different driver mutations, but was able to discriminate between MPN patients and controls. Furthermore, this analysis revealed activation of the JAK–STAT pathway as a central feature in MPN patients, indicating the central role for this pathway in such patients regardless of which driver mutation was present.

- JAK2 mutations

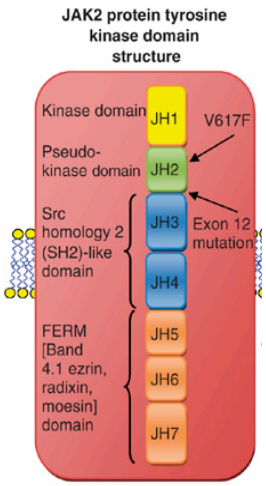


Fig.5: Structure of the cytosolic tyrosine kinase JAK2.The V617F mutation is located in the pseudo kinase domain.

JAK2 is the member of the Janus tyrosine kinase family that is responsible for signal transduction by the erythropoietin, thrombopoietin, granulocyte macrophage colony stimulating factor, and granulocyte colony-stimulating factor receptors in hematopoietic cells, as well as for signal transduction by many cytokine receptors, such as interleukin IL-3, IL-5, and IL-6 receptors (Verma et al., 2003). JAK2 is normally activated when these receptors bind their cognate ligands. In 2005, the first recurrent molecular abnormality has been identified in this gene: the *JAK2V617F* mutation. It is a result of a G to T nucleotide shift at nt 1849 in exon 14, (a point mutation resulting in a switch from phenylalanine to valine at amino acid position 617 (V617F)) that is located in the tyrosine kinase-like domain-2 (JH2), pseudo-kinase domain of JAK2, and likely results in the loss of auto-inhibitory control and/or cytokine-induced hyper-activation of JAK2 (Kundrapu et al., 2008) (Fig. 5).

Expression of V617F-mutated allele in cytokine-dependent cell lines conferred cytokine independence and cytokine hypersensitivity through the constitutive activation of signal transducers and activators of transcription-5 (STAT5),

v-akt murine thymoma viral oncogene homolog 1 (AKT) and extracellular signal-regulated kinase (ERK)- dependent pathways (Levine et al.,2005; Vainchenker W et al., 2013) (Fig. 6); in addition, mice transplanted with marrow cells transduced with a retrovirus expressing *JAK2V617F* invariably developed erythrocytosis (James et al., 2005; Wernig et al., 2006; Lacout et al., 2006; Bumm et al., 2006; Zaleskas et al., 2006) eventually associated with leucocytosis, splenomegaly and later changes suggestive of transformation to post-polycythemic myelofibrosis (Wernig et al., 2006; Lacout et al., 2006; Bumm et al., 2006; Zaleskas et al., 2006).

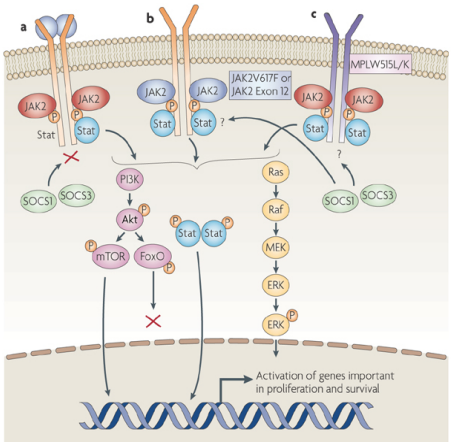


Fig. 6: Mechanism of activation of JAK2 Kinase activity by mutations in the JAK2 signalling pathway.

More recently, transgenic mice presenting an expression of mutated allele lower than wild-type one have been generated, and found to develop an ET-like phenotype (Tiedt et al., 2008; Shide et al., 2007); overall these data suggest that the *JAK2V617F* mutation is an integral component of the myeloproliferative process that underlies the different classic MPNs.

The frequency of *JAK2V617F* mutation is estimated at over 95% in PV, 60% in ET or PMF (Verstovsek et al. 2006; Vizmanos et al. 2006) as well as 50% in patients with the infrequent entity 'refractory anaemia with ringed sideroblasts and thrombocytosis' (RARS-T). The mutation can be found on one or both alleles (homozygosity) as a result of a mitotic recombination process that occurs in most patients with PV or PMF and a minority only of ET (James et al., 2005; Levine et al., 2007; Kralovics et al. 2005) Additional complex mutations, deletions or insertions have been detected in exon 12 of JAK2 and are usually associated with clinical features typical of a JAK2V617F-negative PV or idiopathic erythrocytosis (Scott et al., 2011).

The activated kinase promotes cell proliferation and resistance to cell death, as well as hypersensitivity to hematopoietic growth factors and cytokines signaling through receptors utilizing JAK2. Thus, cells expressing the mutant JAK2 have both a growth and a survival advantage over their normal counterparts and expand their numbers autonomously.

This effect is most pronounced in maturing hematopoietic cells, which require erythropoietin, thrombopoietin, and granulocyte colony-stimulating factor for proliferation and survival, compared with undifferentiated hematopoietic stem cells, which do not (Parganas et al., 1998) and in which only the thrombopoietin receptor is

expressed (Solar et al., 1998).

The presence of *JAK2V617F*, as mentioned in the previous paragraphs, is now included as a major criterion in the revised World Health Organization (WHO) diagnostic criteria for PV, where it is considered as a clonal marker for ET and MF (Tefferi et al., 2008); furthermore, JAK2 mutational load has been suggested to be correlated with the phenotypes of disease.

The *JAK2V617F* mutation can be present in a heterozygous state or can progress to homozygosity most frequently by a mitotic recombination event resulting in uniparental disomy. Different studies suggested that in ET and PV, a higher allele burden is associated to disease progression to MF (Steensma et al., 2006) , but in contrast, a low allele burden is related to an inferior overall and leukemia-free survival in MF (Steensma et al., 2006). It can be an important marker in monitoring the molecular response to the novel JAK2 inhibitors.

The only curative treatment for MF is allogenic stem cell transplantation (ASCT), which was initially associated with significant transplantation-related mortality. Studies reported that *JAK2V617F* mutation disappears after ASCT and reappears in the case of ensuring relapse, and the value of this mutation as a minimal residual disease marker to guide adoptive immune therapy is well established (Huijsmans et al., 2011). Detection of this mutation status and allele burden is helpful in differential diagnosis, prognosis, disease phenotype, complication,and evolution.

JAK2 is an obligatory kinase for the proliferation and differentiation of erythroid cells and megakaryocytes and, therefore, is a relevant therapeutic target for agents that specifically inhibit its activity. To date, clinical trials are investigating several JAK2 inhibitors in patients with myeloproliferative disorder, and more inhibitors are being developed. The results to date indicate that these inhibitors reduce splenomegaly and alleviate constitutional symptoms, such as night sweats, fatigue, and pruritus, in a reversible manner, suggesting that a substantial inflammatory component contributes to the signs and symptoms of the myeloproliferative disorders (Mesa et al., 2007), possibly through JAK2V617F–activated cytokine receptors. However, these inhibitors have had only a modest impact on the JAK2V617F allele burden, although this may require a longer duration of therapy. At the same time, pluripotent hematopoietic stem cells do not seem to require JAK2 for their survival or proliferation (Parganas et al., 1998), and JAK2 mutations, unlike the BCR-ABL fusion kinase in chronic myelogenous leukemia, are probably not the initiating molecular event in polycythemia vera, essential thrombocytosis, or primary myelofibrosis.

- MPL mutations.

Another recurrent molecular abnormality of MPN is represented by somatic mutations at codon 515 of *MPL*, (Pikman et al., 2006) which, as is the case with *JAK2V617F*, involve early myeloid and lymphoid progenitors. Mutations in this gene are identified one year after the discovery of *JAK2V617F* alteration.

MPL gene (named after myeloproliferative leukemia virus oncogene homolog) consists of 12 exons and it is located on chromosome 1p34; it encodes a 635-amino acids protein, constituted by two cytokine receptor motifs (CRMs – approximately 200 amino

acids each), a 22 residues trans-membrane domain (amino acid 492-513) and an intracellular domain containing two conserved motifs termed Box 1 and Box 2 (Mignotte et al., 1994).

MPL gene is highly expressed in early hematopoietic progenitors and in cells of the megakaryocytic lineage (Kaushansky et al., 2005).

The two most common MPL mutations, which are located in the cytoplasmic juxtamembrane portion, are represented by W515L (a tryptophan to leucine substitution) and W515K (a tryptophan to lysine substitution). They have been detected in 5% to 11% of patients with PMF and in up to 9% of JAK2V617F-negative cases of ET (Beer et al. 2008; Vannucchi et al., 2007). Other unusual MPL mutations (eg MPLW515S, W5151A, and MPLS505N, initially discovered in association with inherited familial thrombocytosis) have also been reported (Williams et al., 2007).

MPLW515L induced both cytokine-independent growth and TPO hypersensitivity in cell lines, resulting in constitutively activated JAK-STAT/ERK/Akt signaling pathways, (Chaligne et al., 2008) and caused a PMF-like disease in mice (Pikman et al., 2006).

At variance with the *JAK2V617F* transplantation model, the disease induced by MPLW515L was characterized by a rapidly fatal course, marked thrombocytosis, leukocytosis, hepatosplenomegaly, and bone marrow fibrosis, all reminiscent of PMF (Pikman et al, 2006). Interestingly in some patients, multiple *MPL* mutations or the coexistence with *JAK2V617F* allele were described (Guglielmelli et al., 2007; Vannucchi et al., 2008; Lasho et al., 2006). The presence of the *MPL* mutations does not seem to have an effect on survival or on leukemic transformation and fibrotic. Cases of PMF with the MPL mutation were associated with older age, low hemoglobin levels and a greater likelihood of addiction to frequent blood transfusions (Tefferi et al., 2010; Guglielmelli et al., 2007) .

- CALR mutations

Calreticulin (CALR) is a developmentally highly conserved, multicompartmental and multifunctional protein best known for its role as a Ca^{+} binding chaperone in the endoplasmic reticulum (ER) lumen (Gold et al., 2010). The CALR-1 gene (the product of calreticulin-2 has only been detected in the testis) is located on human chromosome 19 and mouse chromosome 8, spanning 4.6 and 3.6 kb, respectively, and contains nine exons with a highly conserved sequence (96% amino acid identity) between human and mouse. Mature CALR is a 46-kDa protein that consists of three structurally and functionally distinct domains (Fig.7). One of these, the acidic C-terminal domain, is involved in cellular calcium homeostasis—cells that are deficient in CALR have reduced calcium storage capacity in the ER and overexpression of CALR augments ER calcium retention. The C-domain terminates in a KDEL sequence; KDEL receptors function in the retrieval of KDEL-containing proteins from the cis-Golgi back to the ER. The C-terminus of mutant CALR lacks KDEL which raises the possibility of cellular mislocalization, however, preliminary evidence from localization studies of exogenous overexpression of mutant CALR in target cells, as well as localization studies of myeloid cells from CALR mutated patients has shown that mutant CALR largely retains its localization within the ER (Nangalia et al., 2013; Klampf et al., 2013). Within the lumen

of ER, and in concert with other chaperone proteins, CALR plays an essential role in ensuring proper protein and glycoprotein folding. CALR has structural homology with calnexin and both function in concert in the so-called "calnexin/calreticulin cycle", a N-glycan-dependent quality control process that ensures correct glycoprotein folding and/or degradation and prevents protein aggregation (Caramelo et al., 2008). Amongst other roles, *CALR* is implicated in the assembly and cell surface expression of major histocompatibility complex (MHC) class I molecules. Disruption of *CALR* is embryonically lethal with the embryos showing markedly decreased ventricular wall thickness and intertrabecular recesses in the ventricular walls. This appears to be due to impaired myofibrillogenesis as a result of abnormal ER calcium availability. CALR-mediated regulation of calcium homeostasis may influence multiple additional cellular functions including integrin-mediated signaling. In addition to canonical ER related functions, CALR is reported to be found in the cytosol, nucleus, at the cell surface as well as extracellularly, where it accomplishes multiple functions as a critical mediator of physiological and pathological processes such as the immune response, proliferation and apoptosis, cell phagocytosis, wound healing, and fibrosis (Gold et al., 2010). Calreticulin (*CALR*) gene was first recognized as a somatic mutation in patients with MPNs who had no mutations in either *JAK2* or *MPL* by Klampfl *et al.* (Klampfl et al., 2013) and Nangalia *et al.* (Nangalia et al., 2013) in 2013. Ever since this discovery, its molecular and pathogenic roles, and its clinical significance have been the hot research topic in the field of hematopathology. All the mutations identified so far occur in exon 9, with the exception of a few non-recurrent point mutations (Wu et al., 2014), almost all these mutations are indels. More than 50 different *CALR* indels have been described. Klampfl *et al.* has defined those with a 52-bp deletion (p.L367fs*46) as type 1 mutation and those with a 5-bp TTGTC insertion (p.K385fs*47) as type 2 mutations.

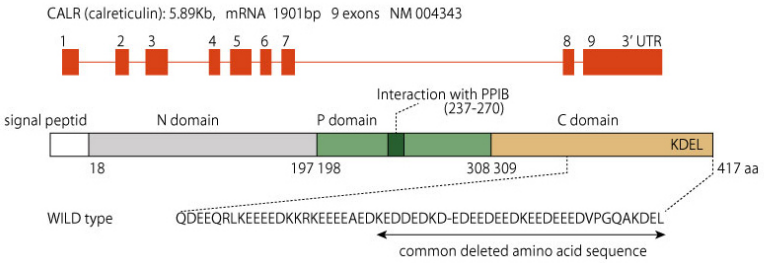


Fig.7: CALR gene structure and its encoded protein.

They have found that these two types of mutations accounted for more than 80% of all *CALR*-mutant patients and the other mutation types including type 3, 4, 5, and 11 were observed at much lower frequencies, many of which were detected only in a single patient (Klamfl et al., 2013). Interestingly, the frequency of type 1 mutation is significantly higher in PMF than in ET, suggesting a specific role of this mutation in myelofibrotic transformation (Rumi et al., 2014). Most of the mutations are heterozygous mutation. Homozygous *CALR* mutations are very rare and they are all 5-

bp insertions (Nangalia et al., 2013). Due to the high prevalence of *CALR* mutation in *JAK2*V617F- and *MPL*-non-mutated patients, it has been suggested that the presentence or absence of *CALR* mutation be added to the British Committee for Standards in Haematology criteria for the diagnosis of ET and PMF. However, using *CALR* mutation for risk stratification or proposed International Prognostic Score for ET (IPS-ET) is not recommended. Since these uses have not been tested and the IPS-ET score was tested upon retrospective selected groups of patients who were already receiving treatment (Harrison et al., 2012). Similarly, a revision of WHO diagnostic criteria for MPNs has been proposed, too (Tefferi et al., 2014). The *CALR* mutations are frequent in *JAK2/MPL*-unmutated ET/PMF and thus provide a much needed clonal marker in such cases. In the context of consistent morphology, *CALR*, as well as *JAK2* and *MPL* mutations, is relatively specific to MPN and should therefore be separated from 'other' clonal markers such as abnormal karyotypes. However, *CALR* mutations do not fully address the molecular gap in *JAK2/MPL*-unmutated disease or distinguish between ET and early/prefibrotic PMF. As such, their availability does by no means undercut the necessity of bone morrow morphology as a major diagnostic criterion in both ET and PMF (Tefferi et al., 2014).

2.3.2 OTHER GENE MUTATIONS FREQUENTLY OCCURRING IN MPN.

Approximately 5-10% of MPNs are triple negative for *JAK2*, *MPL*, and *CALR*. Mutations in many other genes including genes involved in *JAK-STAT* pathway, such as *LNK* and *CBL*, genes involved in epigenetic regulation such as *ASXL1, DNMT3A, TET2, EZH2, IDH1, IDH2*, as well as in genes involved in mRNA splicing, such as *SRSF2 h*ave also been described in recent years in patients with MPNs (Nangalia et al., 2014). It is not clear whether they explain all the MPNs when all these mutations are taken into account. It is also not clear whether these mutations are mutually exclusive or whether they are the drivers of the disease. The mutations in these genes are discussed as follows.

-Mutations in other genes involved in JAK-STAT pathway: LNK and CBL mutations

Over the years following the discovery of the mutation in the *JAK2* and *MPL* , as already mentioned they have been identified in patients with PMF new alterations present in two other genes that are involved in the signaling pathway JAK-STAT : *LNK* and *CBL* .

LNK, also called SH2B3, is a member of the SH2B family, which contains 2 other members. This family of adaptor proteins is characterized by a conserved structure with 3 main domains (a proline-rich amino terminus, a plekstrin homology domain, an SH2 domain) and a conserved tyrosine at the C-terminus. ***LNK*** plays an important role in hematopoiesis by negatively regulating JAK2 activation through its SH2 domain, thus

inhibiting EPO-R and MPL signaling (Tong et al, 2005; Tong et al., 2004). In addition, LNK negatively regulates c-KIT and FMS signaling (Simon et al). LNK-deficient mice have an increased HSC pool with enhanced self-renewal properties and increased quiescence (Takaki et al., 2002).

This phenotype probably results from increased TPO/MPL signaling (Seita et al.2007) because TPO is required for maintaining HSC quiescence and the HSC reservoir (Velazquez et al., 2002). In addition, Lnk -/- mice develop MPN with thrombocytosis, splenomegaly, and fibrosis (Velazquez et al., 2002) and marked B-cell overproduction.

As expected from its negative role in JAK2 signaling, LNK is also capable of attenuating the signaling induced by MPLW515L or JAK2V617F (Gery et al., 2007). Loss of LNK accelerates the development of MPN induced by *JAK2V617F* in murine models (Bersenev et al., 2010).

In *JAK2V617F* positive patients, LNK expression is increased and modulates the myeloproliferative process (Baran et al., 2010). In 2010, Oh et al identified 2 mutations in LNK exon 2, one in a patient with PMF and the other with ET; both MPNs were JAK2V617F negative. The first mutation leads to a premature stop codon resulting in the absence of the PH and SH2 domains, whereas the second (E208Q) is a missense mutation in the PH domain. In the first mutation, the capacity to inhibit TPO signaling is lost, whereas in the second mutation, some inhibitory function is maintained. The frequency of mutations in **LNK** is low (Oh et al., 2010). However, other mutations of LNK have been found in leukemic transformation of MPN at a greater frequency (13%) (Pardanani et al., 2010). All mutations, except one, target a hot spot located between codons 208 and 234. Interestingly, some of these mutations appear to be late events involved in disease progression because they were not found in the chronic phase (Pardanani et al., 2010). In addition some LNK mutations were associated with *JAK2V617F*, although it is not known whether LNK mutants and JAK2V617F were present in the same cell. It has been also reported that LNK exon 2 mutations can be found in pure erythrocytosis (Lasho et al., 2010). One mutation (A215V) had been previously described in PMF blast crisis, and another (E208X) leads to absence of the PH and SH2 domains as the mutant described in PMF (Oh et al., 2010). This finding may suggest that the phenotype of the MPN induced by LNK mutations may depend on different parameters, including the presence of other mutations.

The ***CBL*** protein is a regulator of signal transduction, which acts by various mechanisms. In the JAK-STAT pathway, it can act as an E3 ubiquitin ligase, which can target JAK2, as well as the erythropoietin receptor and thrombopoietin receptor (MPL) (Saur et al., 2010) and (Schmidt et al., 2005) for degradation. **c-CBL** gene is located at 11q.23.3 and is mutated in a variety of myeloid malignancies (Bacher et al., 2010; Kales et al., 2010). The greatest frequency of mutations is found in CMML and juvenile myelomonocytic leukemia. In acute myeloid leukemia (AML), the transforming activity of c-CBL may be related to an increased FLT3 signaling (Rathinam et al., 2010). Usually variants are missense mutations, which are homozygous because of an acquired uniparental disomy (Ogawa et al., 2010) or, rarely, because of a deletion of the wild-type copy. For these reasons, CBL has been considered a tumor suppressor

gene. In fact, most mutated CBL forms behave as loss-of-function molecules having a dominant-negative effect on c-CBL, leading to an excessive sensitivity to a variety of growth factors (Sanada et al., 2009). Retroviral transplantation assays with mutated c-CBL induce a mastocytosis phenotype and myeloid leukemia, albeit with a long latency. Similarly, c-CBL knockout mice develop a mild MPN with an increase in HSC (Naramura et al., 2010). In the chronic phase of classic MPN, c-CBL mutations have been found in a low percentage of PMF patients (6%) but were not detected in a small series of PV and ET patients (Grand et al., 2009). In one case, the c-CBL mutation occured after *JAK2V617F*. However, during progression of the disease, *JAK2V617F* was outcompeted by the CBL mutant, suggesting that the 2 mutations had occurred in 2 different cells (Grand et al., 2009). Similarly a **c-CBL** mutation has been detected in blasts from a *JAK2V617F*-positive MPN, which became *JAK2V617F* negative during transformation (Beer et al., 2008). Presently, **c-CBL** seems to be involved more in progression toward myelofibrosis or acute leukemia than in the chronic phase of the disorder, but further studies are required to establish its precise role.

2.3.3 MUTATIONS IN GENES INVOLVED IN EPIGENETIC MECHANISMS.

Regulation of transcription can be achieved through epigenetic mechanisms, which involve DNA and histone modifications. The most common mechanisms of transcriptional repression are methylation and hidroxymethylation of cytosines in DNA and methylation, acetylation and other modifications of histones.

Several recurrent mutations outside of the JAK–STAT pathway, involving epigenetic modifiers as well as splicing factors, have been identified in MPN patients and appear to have prognostic significance. The genes encoded epigenetic modifiers in which it was demonstrated the presence of somatic mutations in myeloid neoplasms are: ASXL1, TET2, IDH1/2, as well as members of the polycomb repressor complex 2 (PRC2) and DNMT3A (Fig. 8).

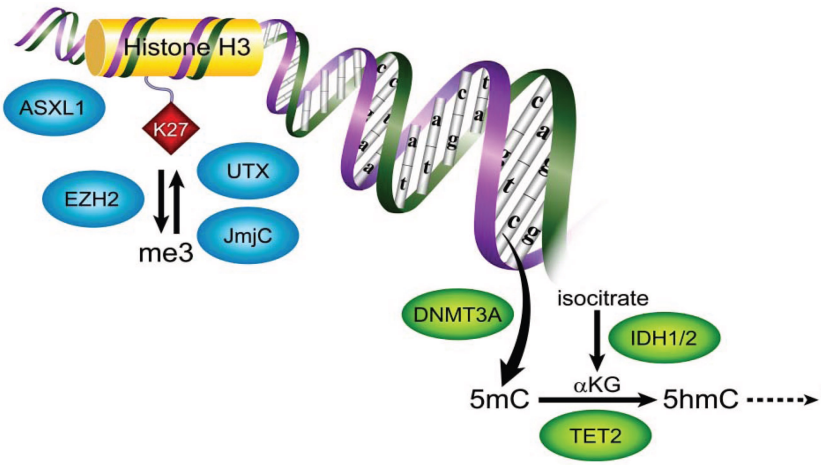


Fig.8: Genetic alterations of epigenetic pathways in MPN. The normal function of selected factors important for histone modification and DNA methylation is depicted. Left panel: In most cases, the biological consequences of mutations in these pathways is not yet well-established. Trimethylation (me3) of lysine 27 (K27) on the carboxyterminal tail of histone H3, a nucleosome component, is associated with transcriptional repression. *ASXL1* is a polycomb repressive complex protein that maintains the repressive state. Right panel: Cytosine methylation in CpG islands is associated with transcriptional repression. DNMT3A is a de novo DNA methyltransferase that converts unmethylated cytosine to 5mC. 5mC is converted to 5hmC by the TET proteins in the presence of alfaKG generated by the IDH enzymes (Graubert et al., 2011).

In MPNs these mutations occurs most frequently in patients with PMF or progression to acute leukemia.

-TET2 mutations

The Ten-Eleven-Translocation2 (TET2) gene codes for a 2-oxoglutarate and Fe(II)-dependent hydroxylase that is able to hydroxylate methylated cytosine (methylcytosine; mC) (Ito et al., 2010). The function of the resulting modified nucleotide, hydroxymethylcytosine (5hmC), may alter chromatin structure and restrict access to DNA methyltransferases, which mediate repression of gene transcription. It follows that loss of TET enzyme activity results in increased methylation.In fact, observed mutations are mainly small insertions and deletions and nonsense mutations that are expected to result in the loss of function of the protein. Missense mutations affecting conserved amino acids have been shown also to impair the catalytic activity of the protein. Accordingly, patients with ***TET2*** mutations have lower global 5hmC content than wild type (Ko et al., 2010).

TET2 is mutated in a wide range of myeloid malignancies (Langermeijer et al., 2009).

TET2 mutations are found in approximately 14% of MPNs ranging from ET (11%) to PMF (19%) (Tefferi et al., 2009). In roughly 20% of the patients, 2 mutations are observed, suggesting that the inactivation of a single copy of *TET2* is sufficient for the transformation process. ***TET2*** mutations are not responsible for the familial MPNs identified to date, although a germline mutation has been described. In vitro studies have first shown that *TET2* mutations occur before *JAK2* mutations during the natural history of sporadic MPNs (Delhommeau et al., 2009). However, subsequent studies suggest that the converse may happen and also that *TET2* mutations may occur when MPNs transform to AML (Schaub et al., 2010; Swierczek et al., 2011; Saint-Martin et al., 2009; Abdel-Wahab et al., 2010).

- IDH1/2 mutations

IDH1/2 **(Isocitrate Dehydrogenase 1/2):** *IDH1/2* are homodimeric $NADP^+$ dependent enzymes involved in the conversion of isocitrate to α-ketoglutarate by oxidative decarboxylation prior to NADPH synthesis (Dang et al., 2010). Heterozygous mutations of these genes were first described in gliomas and secondary glioblastomas and then in AML (Okita et al., 2012; Abbas et al., 2010). Mutations in *IDH1/2* can lead to an increase in NADPH-dependent reaction of α-ketoglutarate resulting in over production of α-hydroxyglutarate which is a potential toxic substance and conversely have a negative effect on the function of the *TET2* protein (Figueroa ME et al., 2010). Both *IDH1/2* mutations and *TET2* mutations lead to similar hypermethylation signatures and patterns of impaired myeloid differentiation and increased expression of stem cell markers. *IDH1/2* mutations usually involve the amino acid R132 (IDH1), R140 and R172 (IDH2). The frequency of *IDH1/2* mutation is about 3-5%, 1-2%, and 10-20% in PMF, post-PV/ET MF and in MF cases evolving to AML (Pardanani et al., 2010). Also, an analysis of 1473 patients with MPN revealed a low incidence of *IDH1/2* mutations in chronic phase ET, PV, and MF (0.8%, 1.9%, and 4.2%, respectively) contrasting with a

21.6% frequency in blast phase (Pardanani et al., 2010). Thirtyeight *IDH1/2* mutations have been discovered in a large screening study of MPN patients and can coexist equally with mutations in *JAK2, MPL,* and *TET2* (Skoda, 2007). Over 21% of patients with blast phase-related MPN carry an *IDH1/2* mutation, and this was irrespective of *JAK2V617F* status (Pardanani et al., 2010; Green et al., 2010). This appears to indicate that *IDH1/2* mutations can also influence the transformation of MPN to blast phase disease. Interestingly, leukemic blasts and progenitor cells can possess both mutated *IDH2* and *JAK2V617F*, and in other patients with MPN-transformed leukemia, the mutated *IDH1/2* may be present in blasts with wild-type *JAK2* and absent in the progenitor cells with *JAK2V617F* (Green et al., 2010). These data have shown the possibility of the presence of two subclones originating from a yet unidentified primary clone or two independent competing clones arising in the same individual.
Furthermore, mutations in these genes are usually observed to be mutually exclusive from mutations of *JAK2*, *TET2*, and *MPL* (Tefferi et al., 2011).
Also, in a more recent study of IDH1/2 mutations in a cohort of 301 patients with PMF, it was shown that IDH1/2 mutations are associated with decreased overall survival and leukemia-free survival in PMF (Dang et al., 2010).

- ASXL1 mutations

ASXL1 *gene* encodes the *A*dditional *SeX* combs–*L*ike protein-*1* which is one of the three mammalian homologs of *Drosophila* Additional Sex Comb (*Asx*) gene, named after the fact that *Asx* deletion caused homeotic transformation due to dysregulation of *Hox* genes, whose spatially and quantitatively appropriate expression is essential for the anterior-posterior specification of axial structures during mammalian development. *ASXL1* maps to human chromosome 20q11.21, consists of 12 exons and encodes a protein composed of 1,541 amino acids. Mutations in ***ASXL1*** are frameshifts and stop mutations located within the 12th exon of the gene; they usually affect only one copy of the gene and result in the loss of the carboxyterminal PHD domain. The frequency is low in low-grade disorders but greater in late MDS, AML, and in proliferative CMML. In MPNs, *ASXL1* mutations are rare in ET and PV (7%) but frequent in PMF (from 19%-40%) (Ricci et al., 2012; Carbuccia et al., 2009). The function of ***ASXL1*** in hematopoiesis is still poorly understood. ***ASXL1*** knockout mice have a mild defect in hematopoiesis, predominantly in lymphopoiesis (Fisher et al., 2010). A marked decrease in myeloerythroid progenitors was observed but without detectable effect on HSC. Its role in human hematopoiesis is unknown.
Finally ,a large study conducted on patients with PMF has established that the presence of mutations of *ASXL1* is associated to a phenotype aggressive is to a reduced survival (Guglielmelli et al., 2011) but in that way the mutations of ***ASXL1*** determine a phenotype of disease more aggressive not is again the all clarified.

-DNMT3A mutations

The ***DNMT3A*** heterozygous mutations were initially described in AML and MDS with a frequency of 5-22% (Lin et al., 2011). However, mutations have been found, also, in

gene ***DNMT3A*** in over the 15% of patients with MF and in over the 7% of patients with PV (Abdel - Wahab et al., 2011) and ET (Stegelmann et al., 2011).
This gene encodes a DNA methyltransferase that is essential in *de novo* methylation. Acquisition of mutations can cause loss-of-function resulting in homo-dimerization and activation of the protein, reduction of the activity of methyltransferase, and consequent increased cell proliferation (Stegelmann et al., 2011).
The somatic mutations in this gene are nonsense , frameshift , and missense in the open - reading frame . However , the mutations more frequently lead a premature break of the product protein (nonsense or mutations frameshift) or they affect a single amino acid : R882 . *DNMT3A* can play a significant role in progression of MPN to AML in the presence of *JAK2* and *MPL* mutations. It has been showed that the time of mutational event acquisition reflects the disease course. In order to further dissect the role of this gene in MPN, a study described the isolation of different cellular lineages and the successive assessment of the cell type harboring high frequency of *DNMT3A* mutations. Mutations were identified with high frequency in $CD14^+$ (monocytes) enriched fraction and with low frequency in $CD3^+$ and $CD19^+$ (T and B lymphocytes, respectively) suggesting that the aberrant clone does not occur in lymphoid lineages (Rao et al., 2012). Indeed, *DNMT3A* mutations are associated with over expression of other relevant genes in advanced myeloid malignancies (Stegelman et al., 2011).

-EZH2 mutations

This gene is located in chromosome 7 (7q36.1) and belongs to the complex 2 of Polycomb, a mediator of transcriptional silencing and a regulator of multiple cellular process like proliferation, maturation, aging and hematopoietic cell plasticity (Shen et al., 2008). *EZH2* over-expression is noted in a variety of solid tumors like prostate and breast cancer and it has been reported to contribute to tumor aggressiveness and poor cellular differentiation. (He et al., 2010). *EZH2* mutations appear to be a gain-of-function genetic change acting as a repressor by methylating the histone H3 on lysine 27 (H3K27) and consequently inactivating the chromatin (Cao et al., 2002). The frequency of *EZH2* mutations is approximately 12% in MF; however, mutations are not exclusively found in MPN since they are more frequent in MDS (2-6%) and MDS/MPN (~15%). Clinically, *EZH2* mutations have been associated with poor prognosis (Ernst et al., 2010).

-SRSF2 mutations

A other gene that results changed in 17% of patients with PMF is *SRSF2*. It results changed also in 18.9% of patients with leukemia secondary to MPN and in 28% of cases is associate to mutations of *IDH1/2*. The mutations of *SRSF2* have been found in phase chronic and leukemic from themselves patients and they are associated to prognosis unfavorable (Lasho et al, 2012; Zhang et al., 2012). Studies latest have taken into consideration the possible prognostic maening of some of these mutations.
This status established that a set of four mutations including the genes *ASXL1, EZH2, SRSF2* and *IDH1/2* allow to identify patients to high risk molecular (Category High Molecular Risk - HMR, or patients that have changed at least one of 4 genes),

independently from category of risk IPSS (Vannucchi et al., 2013). The condition HMR is associated to a hazard ratio (HR) for the survival of 2.29 (95% CI, 1.6-3.2) and of 2.96 (95% CI, 1.8-4.7) for the risk of blast evolution. About the 20% of patients considered to bass risk according to the score IPSS have a profile molecular type HMR, is could then require a approach therapeutic different by that currently employed. More recently Guglielmelli et al. have observed that in patients that have two or more of mutations including in category HMR the value HR for the survival, in analysis multivariate stratified for the score IPSS, is of 2.4 (1.6-3.6) while for the evolution blast is of 6.2 (3.5-10.7) (Guglielmelli et al., 2014). Despite the fact that the mutational background of MPNs has been extensively investigated, the molecular etiology of the disease has not been fully elucidated. Indeed several lines of evidence indicate that the identified mutations are not sufficient for disease initiation and progression.

2.4 OTHER POSSIBLE EPIGENETIC MECHANISMS INVOLVED IN PATHOGENESIS OF MPN: microRNAs.

Early studies have shown how aberrantly expressed microRNAs are a hallmark of several diseases like cancer. MicroRNA expression profiling was shown to be associated with tumour development, progression and response to therapy, suggesting their possible use as diagnostic, prognostic and predictive biomarkers.

2.4.1 WHAT IS A MIRNA?

MiRNAs were defined as noncoding RNAs that fulfill the following combination o expression and biogenesis criteria:

1. mature miRNA should be expressed as a distinct transcript of 18–24 nt that is detectable by RNA (northern) blot analysis or other experimental means such as cloning from size-fractionated small RNA libraries;
2. mature miRNA should originate from a precursor with a characteristic secondary structure, such as a hairpin or fold-back, that does not contain large internal loops or bulges. Mature miRNA should occupy the stem part of the hairpin;
3. mature miRNA should be processed by Dicer, as determined by an increase in accumulation of the precursor in Dicer-deficient mutants.

In addition, an optional but commonly used criterion is that mature miRNA sequence and predicted hairpin structure should be conserved in different species. An 'ideal' miRNA would meet all the above criteria. In practice, variations are possible, but at the very minimum expression of a ~18-24 nt form and the presence of a hairpin precursor need to be demonstrated to classify a sequence as an miRNA.

MiRNAs are antisense RNA molecules that , thanks to their characteristics , they work by guiding elements in post- transcriptional gene silencing. Indeed matching to target mRNAs it promotes their degradation or as happens more frequently, block its translation.

2.4.2 THE DISCOVERY OF MIRNAS.

MiRNAs control the expression of genes involved in several biologic processes such as apoptosis, proliferation, differentiation and metastasis .

Two decades ago, the existence and importance of miRNAs was completely unknown. Until then, the scientific community was focused on genes that codify for protein. The classical dogma that DNA is transcribed into RNA which then is translated into protein put aside the study of all the non-protein coding sequences. Only in 1993 the importance of miRNAs started to be revealed. In fact, in December 1993, in the same issue of Cell, Ambros and Ruvkun independently reported that the small and non-protein coding transcript lin-4 in the *Caenorhabditis elegans* regulates lin-14 through its 3' UTR region (Lee et al.,1993; Wigthman et al.,1993): Lin-4 was the first miRNA to be discovered. For some years Lin-4 appeared to be an isolated case until the discovery of another miRNA gene, again in *C. elegans*, known as let-7. The discovery that let-7 is conserved across species triggered a revolution in the research of a new class of small ncRNAs, called miRNAs. Presently, thousands of miRNAs had been identified in humans and other species, and miRNA online sequences repositories, such as the miRbase database, are available (Griffiths et al.,2004; Griffiths et al., 2006; Griffiths et al., 2008). Also, current tools and software developed for miRNA target prediction facilitate studies of miRNAs functional network (Xia et al., 2009; Huang et al., 2009).

2.4.3 MIRNA BIOGENESIS.

The biosynthesis of miRNAs is a tightly regulated multistep process that starts in the nucleus of the cell, following transcription, and continues through the cytoplasm where finally the mature miRNA molecule exerts its main function. miRNA genes can be grouped on the basis of their genomic locations:

1) exonic miRNA in non-coding transcription units; 2) intronic miRNA in non-coding transcription units; and 3) intronic miRNA in protein-coding transcription units. Mixed miRNA genes can be assigned to one of the above groups depending on the given splicing pattern. The biogenesis process begins with the synthesis of a long transcript known as primary miRNA (pri-miRNA) (Fig.9) (Ozsolak et al., 2008; Corcoran et al., 2009).

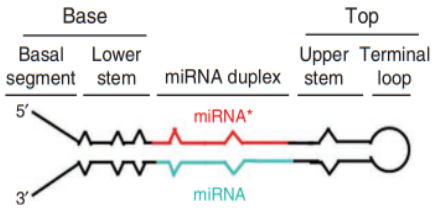


Fig.9: Pri-miRna structure.

Pri-miRNAs are typically (although not exclusively) transcribed by RNA polymerase II, which generates the pri-miRNA that consists of one or more hairpin structures, each composed of a stem and a terminal loop. Pri-miRNAs are structurally analogous to mRNAs; they are 5'-capped and spliced and bear a 3' poly-A tail, and they often can produce more than one functional miRNA (Carthew et al., 2009; Kim et al., 2009; Cai et

al., 2004; Lee et al., 2004). However, other pathways generate a minor set of miRNAs, especially from genomic repeats (i.e., Alu repeats) whose transcription is carried out by RNA polymerase III. (Borchert et al., 2006).

In the canonical miRNA biogenesis pathway the first step of miRNA maturation starts in the nucleus where the pri-miRNA is "cropped" into a ~70 nt hairpin structured pre-miRNA. This phenomenon is catalyzed by a multiprotein complex called the microprocessor. The core components of this complex are Drosha, an RNase III enzyme, together with its interacting partner DGCR8 (DiGeorge syndrome critical region gene 8), a double-stranded RNA-binding domain (dsRBD) protein (Lee et al., 2003; Denli et al., 2004; Gregory et al., 2004). The microprocessor appears to represent a heterotetramer consisting of two Drosha and two DGCR8 molecules. Following nuclear processing by Drosha the pre-miRNA is recognized by exportin- 5 and transits to the cytoplasm to be further processed (Lund et al., 2004; Yi et al., 2003). Owing to compartmentalization of the processing events, nuclear export of pre-miRNAs is a crucial step in miRNA biogenesis.

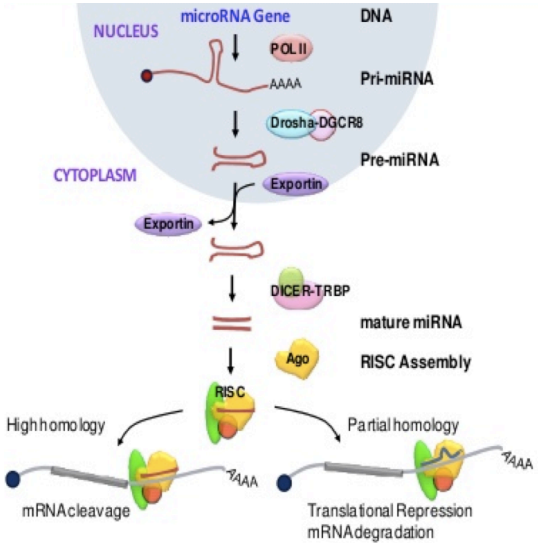


Fig. 10: Biogenesis of miRnas.

Following its export from the nucleus, the pre-miRNA is cleaved by another RNase III DICER, which results in a 18-24 nucleotide miRNA: miRNA* duplex (Bernstein et al., 2001). The duplex is then unwound and one strand is associated with the Argonaute family of proteins which is the central component of the RNA induced silencing complex (RISC). Once incorporated into the RISC, the single-stranded mature miRNA will guide the RISC to interact with the target mRNA via the latter's 3'-untranslated region (Fig.10). Recently, DROSHA-independent or DICER-independent production of miRNAs has been reported (Ruby et al., 2007; Cheloufi et al., 2010; Cifuentes et al., 2010).

2.4.4 MICRORNA–OFFSET RNAS (MORNAS).

Recent studies have exponentially increased the number of known noncoding RNA categories and short RNA sequencing led to the discovery of a novel type of miRNA related small RNA, miRNA–offset RNA (moRNA), whose function is currently unknown. MoRNAs were first reported in a simple chordate, the ascidian Ciona intestinalis, as ~20-nt-long RNAs derived from the ends of pre-miRNAs, possibly by RNAse III-like processing. moRNAs can originate from either end of the pre-miRNA, but they are prevalently derived from the 5' arm, regardless of the major miRNA position. This suggests that moRNA and miRNA biogenesis might be linked but not interdependent. The expression levels of moRNAs seem to be regulated in different developmental stages of Ciona, and their abundance can exceed that of the corresponding mature

miRNA (Bortoluzzi et al., 2011).

In addition, short RNA sequencing led to the discovery of moRNAs, which were first reported in a simple chordate, the ascidian Ciona intestinalis (Shi et al., 2009), as ~20-nt-long RNAs derived from the ends of pre-miRNAs, possibly by RNAse III-like processing (Fig. 11).

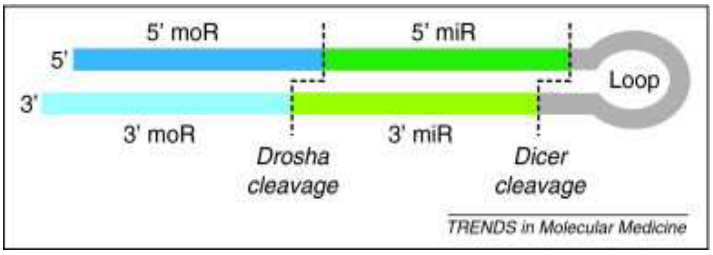


Fig.11: moRNAs. Two miRNAs and two moRNAs can be produced by transcription and processing of a single miRNA locus.

MoRNAs can originate from either end of the pre-miRNA, but they are prevalently derived from the 5'arm, regardless of the major miRNA position. This suggests that moRNA and miRNA biogenesis might be linked but not interdependent. The expression levels of moRNAs seem to be regulated in different developmental stages of Ciona, and their abundance can exceed that of the corresponding mature miRNA.

Initially, moRNAs were considered as by-products of potentially atypical miRNA processing (Shi et al., 2009). Later, moRNAs were also found in human cells by deep sequencing data analysis. Langerberger et al. reported that moRNAs from 78 genomic loci are weakly expressed in the prefrontal cortex (Langenberger et al., 2009). They also observed that some moRNAs are as conserved as miRNAs, and that miRNA precursors that also contain moRNAs are typically old from an evolutionary perspective (Langenberger et al., 2009). Furthermore, some weakly expressed moRNAs have been found in solid tumors, together with other small RNAs (Meiri et al., 2010).

moRNAs are generally included in the miRNA hairpin precursor, and in some cases the moRNA overlaps the miRNA position by a few nucleotides (Langenberger et al., 2009). Other moRNAs that overhang the miRNA hairpin can be produced by non-canonical Drosha processing (Berezikov et al., 2011). Thus, it is not clear how the two ends of moRNAs arise and if or how Drosha and Dicer are involved. moRNAs seem to be conserved across species, the conservation extent correlates with expression level (Shi et al., 2009) and expression levels of certain moRNAs are greater than for their corresponding miRNA (Umbach et al., 2010). In addition, moRNAs are prevalently produced by the 5' arm of the precursor, independent of which arm produces the most expressed mature miRNA (Langenberger et al., 2009; Umbach et al., 2010). This evidence suggests that moRNAs might be miRNA co-products, representing a distinct functional class of miRNA-related agents (Berezikov et al., 2011). The hypothesis that moRNAs are a new class of functional regulators whose qualitative alteration and/or expression dysregulation might impact on human diseases is intriguing, but evidence regarding possible moRNA functions is still fragmentary.

2.4.5 ROLE OF MICRORNAS IN HEMATOPOIESIS

In hematopoiesis a limited number of multipotent hematopoietic stem cell differentiate into cells of all lineages that constitute the blood. This process of

differentiation is well characterised and involves intermediate progenitors with decreasing self-renewal ability and increasing lineage commitment. Lineages are defined functionally and morphologically and lineage commitment is controlled by complex network of transcription factors that define specific gene expression patterns for every cell type. There are several miRNAs that appear to play a role in the most primitive hematopoietic compartments (Fig 12).

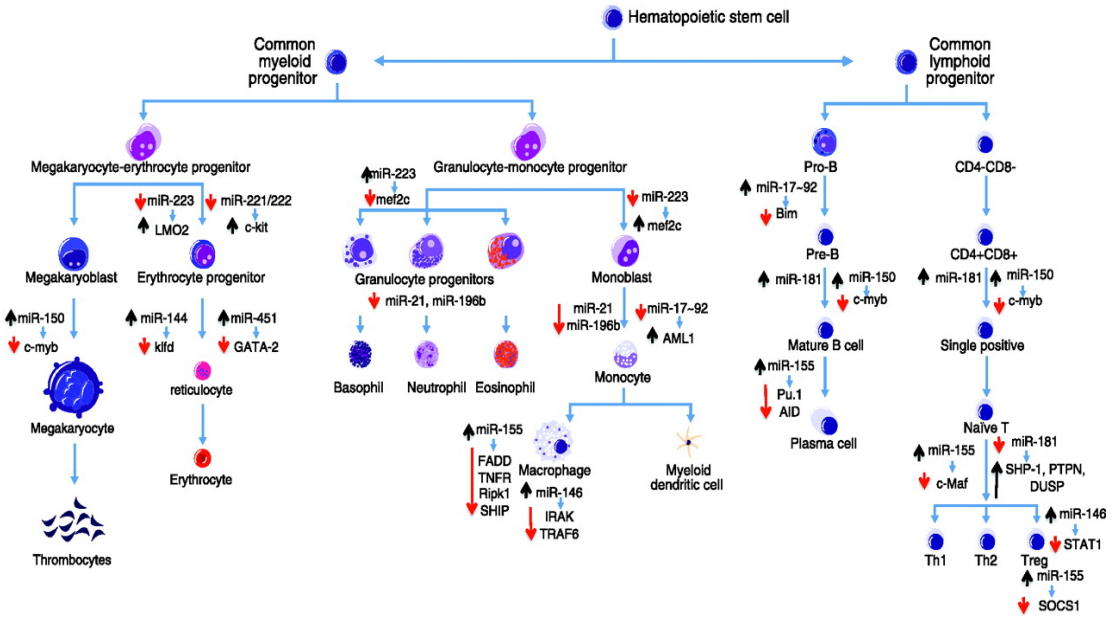


Fig. 12: A diagram showing miRna and their targets that play a role in the development of the hematopoietic system. The diagram lists the various miRnas and their targets that are involved in hematopoiesis.
Upregulation or downregulation of a specific miRna is represented by an upward (black) or a downward (red) arrow, respectively. The changes in the expression levels of a validated target gene inversely correlate with that of the targeting miRna and are similarly represented by an up or down arrow.(Sayed et al., 2011).

The miR-125 family (consisting of three members, miR-125a, miR-125b1 and miR-125b2) is crucial for the maintenance of self-renewal and differentiation balance. As shown by several groups (Gerrits et al.,2012; Guo et al., 2010; Ooi et al., 2010), members of the miR- 125 family are highly expressed in Hematopoietic Stem and Progenitor Cells (HSPCs) and their expression decreases upon differentiation. Enforced expression of these miRNAs in HSPCs provides a proliferative advantage and skews differentiation towards the myeloid lineage (Guo et al., 2010; Chauduhuri et al., 2012) Most recent data clearly show that all miR-125 family members promote increased stem cell self-renewal, but their exact targets are still unknown (Chaudhuri et al., 2012; Wojtowicz et al., 2014). The phenotypes induced by miR-125s maybe partially explained by the inhibition of apoptosis, as miR-125 targets several pro apoptotic genes and genes involved in p53 pathway (Bousquet et al., 2012; Unoli et al., 2001). Many miRNAs reside at introns or within close proximity of protein coding genes and are often co-expressed with these genes (Isik et al., 2010; Li et al., 2012). A good example is the cluster of HOX genes, which encodes members of the miR-196 family. The HOX family of transcription factors is essential for the development and several HOX genes play an important role in hematopoiesis. In the mouse and human

genomes miR-196 bis positioned between HoXA9 and HoXA10 genes. Expression of miR-196b correlates with the expression pattern of HOXA9, and is highly expressed in HSPCs. MiR-196b regulates transcription of genes involved in cell survival and proliferation (Li et al., 2012) but also maintains primitive cells at an undifferentiated state by repressing genes involved indifferentiation (Yekta et al., 2004). The balanced expression of HOX genes is controlled by miR-196b which represses HOX genes that are normally upregulated during myeloid differentiation (Yekta et al., 2004). Other HOX genes involved in hematopoietic differentiation such as HOXA7 and HOXC8 were found to contain sequences complementary to the miR-196b seed sequence and have been confirmed as direct targets (Yekta et al., 2004). More evidence for miR-196b's role in suppressing differentiation comes from the observation that the transcriptional repressor Gfi1 required for myeloid differentiation down-regulates miR-196b during myelopoiesis (Velu et al., 2009). The miR-17–92 cluster is transcribed as a single non-coding RNA that is subsequently processed into seven mature miRNAs: miR-17-5p, miR-17-3p, miR-18a, miR-19a, miR-19b, miR-20a and miR-92a. Some miRNAs in the miR-17-92 cluster share expression patterns which is often the case for miRNA clusters as they are typically under control of the same promoter (Baskerville et al., 2005; Jin et al.,2008). It has been shown that four of the eight miRNAs were higher expressed in hematopoietic stem cells compared to mature blood cells (miR-19a,miR-19b, miR-20a and miR-20b) (Jin et al., 2008) and overexpression of the entire cluster leads to stem cell expansion (Li et al., 2012) . As expected, transcription factors targeted by miR-17-92 miRNAs are involved in differentiation and cell cycle regulation (Xiao et al., 2008). Yet overexpression of individual miRNAs induced various blood malignancies (Li et al., 2012) high lighting the importance of the coordinated regulation of multiple targets involved in similar pathways for normal HSPC maintenance and self-renewal.

-MicroRNAs involved in the regulation of erythropoiesis and megakaryopoiesis.

Several miRNAs have been implicated in the regulation of erythropoiesis, among which are miR-15a, mir-24, miR-144 and miR-451. In order to enable erythroid differentiation, suppression of the self-renewal of HSPCs and a switch in gene expression to an erythroid signature pattern are imperative. Many of the miRNAs upregulated in erythroid cells target genes that normally promote myeloid lineage differentiation. These include GATA-1 and GATA-2, which are regulated by miRNA-24, miR-27a (Wang et al., 2008; Wang et al., 2014) and miR-451 (Pase et al., 2009). Their interaction with PU.1 is a bifurcation point between myeloid and erythroid lineage commitment (Nerlov et al., 2000). During erythropoiesis, GATA-1 progressively out competes GATA-2, and this particular GATA switch is mediated by miR-144 and miR-451 (Dore et al., 2008). MiR-144 and miR-451 are both upregulated during erythroid differentiation and are transcribed as a single pri-miRNA transcript (Dore et al., 2008). GATA-1 and GATA-2 are both able to bind upstream of miR-144/miR-451 but their ability to activate transcription differs: only GATA-1 activates transcription of miR-144/ miR-451 pri-miRNA.In all reports GATA-1 is involved in a positive regulatory loop with

the miRNAs that regulate the GATA-switch: GATA-1 activates transcription of miRNAs that repress GATA-2 and facilitate binding and transcriptional activation of erythroid genes by GATA-1. One of the suggested targets of miR-451 is c-Myc, a transcription factor and oncogene involved in HSC self-renewal. Consequently, its downregulation may allow for differentiation of progenitors into the erythroid lineage. Interestingly, although initially believed to be primarily expressed in lymphocytes (Xiao et al., 2007) miR-150 appears to also be important for megakaryopoiesis. One of its targets is c-myb that regulates transcription factors such as Kruppel Like Factor(Klf1) and Lmo2, which enhance erythropoiesis (Lorenzo et al., 2011) . MiR-150 is up regulated during megakaryopoiesis but downregulated in erythropoiesis suggesting that its level of expression regulates cell lineage commitment towards differentiating megakaryocyte–erythrocyte progenitors.

-MicroRNAs important for granulopoiesis.

As discussed above, miRNAs can actin positive regulatory loops but additionally, miRNAs are also able to actin negative feedback regulatory pathways and this characteristic aids in the regulation of lineage commitment. An exemplary case is provided by miR-223 (Fazi et al., 2005). During granulopoiesis miR-223 acts in a negative cascade pathway to both repress the erythroid transcription factor NFI-A at the RNA level (Starnes et al., 2009) while at the same time associating with Ying Yang1 (YY1), a member of Polycomb Repressive Complex1 (PRC1). This is quitean atypical association for miRNAs, as they rarely associate with proteins outside of the RISC complex. This provides a good example of how miRNA regulation can occur through binding with other proteins, to form complexes able to repress gene transcription. All of the above proteins bindin proximity of the NFI-A locus to repress it stranscription (Zardo et al., 2012) and collectively lead to enhanced granulopoiesis induced by C/EBP/α (Fazi et al., 2005). While miR-223 transcription is activated by the binding of either NFI-AorC/EBP/α to its promoter,C/EBP/α is the more potent activator. Both transcription factors compete for binding at the miR-223 promoter but they differ in their degree of transcriptional activation. During granulopoiesis, however, NFI-A is unable to compete with C/EBP/α resulting in increasing miR-223 transcription and sustained NFI-A repression (Fazi et al., 2005).

2.4.6 MIRNA AND CANCER.

Three important observations early in the history of miRNAs suggested a potential role in human cancer. Firstly, the earliest miRNAs discovered in the roundworm *C. elegans* and the fruit fly *Drosophila* were shown to control cell proliferation and apoptosis (Lee et al., 1993; Brennecke et al., 2003). Their deregulation may therefore contribute to proliferative diseases such as cancer. Secondly, when human miRNAs were discovered, it was noticed that many miRNA genes were located at fragile sites in the genome or regions that are commonly amplified or deleted in human cancer (Calin et al., 2004). Thirdly, malignant tumors and tumor cell lines were found to have widespread deregulated miRNA expression compared to normal tissues (Calin et al., 2006; Gaur et al., 2007; Lu et al., 2005). The question remained whether the altered

miRNA expression observed in cancer is a cause or consequence of malignant transformation. In 2002, the first direct evidence for an involvement of miRNAs in cancer was reported (Calin et al., 2002). Calin et al. studied a well-known deletion on chromosome 13, which is the most frequent chromosomal abnormality in chronic lymphocytic leukemia (CLL). This deletion had long been suspected to contribute to leukemogenesis. However, extensive studies had failed to identify a causal gene. Calin et al. (Calin et al., 2002) found that two miRNA genes, *mir-15* and *mir-16*, were located within this 30-kb deletion. They subsequently analyzed the expression of miR-15 and miR-16 in blood samples from patients with CLL. Both miRNAs were absent or downregulated in the majority (68%) of cases when compared to normal tissue or lymphocytes. This finding suggested that these two miRNAs were causally involved in the pathogenesis of chronic lymphocytic leukemia. In 2005, three reports provided the first mechanistic insight into how miRNAs might contribute to carcinogenesis. Two independent studies described the relationship between a miRNA cluster, *mir-17-92*, and the Myc oncogenic pathway (He et al., 2005; O' Donnell et al., 2005). A third report demonstrated an interaction between *let-7* miRNA and the RAS proto-oncogene (Johnston et al., 2003).

- Abnormal expression of miRNA in cancer.

Tumor tissues and cultured tumor cells often exhibit significantly reduced expression levels of mature miRNAs (Krutzfeldt et al., 2005). Different mechanisms for the aberrant expression of miRNA were documented. Three of them, (i) genetic alterations and single nucleotide polymorphism (SNP), (ii) epigenetic silencing and (iii) defects in the miRNA biogenesis pathway, are discussed below.

(i) Genetic alterations and SNP: Complete mapping of human miRNA genes revealed that a great majority of the miRNAs were associated with fragile sites, cancer-specific translocation breakpoints, repetitive sequences and CpG islands (Kumar et al., 2007). However, some studies have indicated such association is not straight-forward and appears to be dependent on the specific type of cancer (Lagos-Quintana et al., 2001). Furthermore, the existence of polymorphism in single nucleotides (SNPs) is widely known and, evidence has been presented suggesting the influence of SNPs on miRNA targets in cancer-related pathways (Lai et al., 2005). A gain in function due to SNP may enhance its interaction with miRNA target and thus, enhance its regulatory function such as a tumor suppressor gene. In contrast, loss in function due to SNP may result in increased expression of miRNA, which then acts as an oncogene (Lau et al., 2001). Additionally, SNPs in target sites of miRNAs may also result in the escape of degradation by miRNA (Lee et al., 2001). All these observations suggested that SNPs may be one of the contributing factors in the regulation of biogenesis and functionality of miRNAs.

(ii) Epigenetic regulation of miRNA expression: Several research groups have investigated whether epigenetics, i.e., hyper- or hypo-methylation (an early event in carcinogenesis), play a role and influence the activity of miRNA genes (Lee et al., 1993; Lee et al., 2003; Lee et al., 2007) since the expression of miRNA genes, especially those located near CpG islands, tends to be affected more readily by methylation

processes (Lee et al., 1993; Lee et al., 2003; Lee et al., 2005). In scientific literature, there were several examples of DNA methylation processes influencing the activity of miRNAs. In addition to DNA methylation, histone acetylation was also reported to be another epigenetic phenomenon in deregulated cancers. In breast cancer cells, histone deacetylase inhibition was shown to result in alteration in miRNA levels (Lu et al., 2005). In bladder cancer cells, a combined treatment with 5-aza-2'-deoxycytidine (5-Aza-CdR) and histone deacetylase (HDAC) inhibitor 4-phenylbutyric acid (PBA) had a significant effect on multiple miRNAs among which miR-127 was most differentially expressed (Lee et al., 2005). Specific induction/activation of miRNA-127 by 5-Aza-CdR and PBA suppressed the transcription of the zinc-finger repressor BCL6 gene and thus induced apoptosis in human cancer cells (Lee et al., 2005).

iii) Defects in the miRNA pathway: In humans, the majority of miRNAs are encoded by introns of non-coding or coding transcripts. However, some miRNAs were reported to be encoded by exonic regions. The genes controlling miRNA are often clustered and transcribed as polycistronic messages or excised from mRNAs (Lu et al., 2005). The precise locations of promoters for most miRNA genes are not yet mapped but, they can be inferred from collective analysis of CpG islands, RNA sequencing and chromatin immune-precipitation followed by ChIP-sequencing (Lujambio et al., 2007) . Numerous Pol-II associated transcription factors were reported to activate or repress several miRNA genes. The abundance of some miRNAs were also shown to be regulated at the RNA stability level (Ma et al., 2007).

2.4.7 CIRCULATING MIRNAS AND ROLE IN CANCER.

Previous studies have demonstrated that miRNAs are stable also in serum and plasma and that their expression profile responds to changes under different physiological and pathological conditions (Chim et al., 2008; Gutwain et al., 2005) . Although circulating miRNAs may serve as biomarkers for various types of cancers (Lodes et al., 2009; Lawrie et al., 2008), the isolation and measurement of circulating miRNAs remains a challenging task. In addition, although many studies have attempted to explain the origin and function of circulating miRNAs in cancer patients, no definitive source for these molecules has been proposed. Some cell-free miRNAs in body fluids may be packaged in exosomes, microvesicles or RNA binding-proteins, which provide protection from RNases (Huan et al., 2013; Arroyo et al., 2011; Gibbings et al., 2009; Hunter et al., 2008; Zhang et al., 2010) and enable their transfer from one cell to another during diverse biological processes. By defining tumors as relatively homogeneous cancer cells formed in an independent microenvironment, circulating miRNAs may play a novel role as regulators of cell-cell communications during cancer formation (Hergenreider et al., 2012; Chen et al., 2014). Circulating miRNAs have numerous advantages including stably existing in almost all body fluids, cancer-specific or tightly correlating with physiological and pathological changes and easily detectable. Consequently, a number of studies have proposed that circulating miRNAs could be ideal biomarkers for the prediction and prognosis of cancer. The first comprehensive analysis of circulating miRNAs in cancer patients was performed by Lawrie et al. They compared the expression levels of tumor associated miR-155, miR-210 and miR-21 in

serum from patients with diffuse large B-cell lymphoma with levels in serum from healthy controls and observed higher levels in patients compared with controls (Lawrie et al., 2008). Mitchell et al. established an important approach when measuring tumor-derived miRNAs in serum or plasma and suggested that serum level of miR-141 can distinguish patients with prostate cancer from healthy controls (Michell et al., 2008). In hepatocellular cancer, based on microarray and qRT-PCR, seven circulating miRNAs (miR-21, -26a, -27a, -122, -192, -223, and -801) were reported to differentiate HCC patients from healthy controls, patients with chronic hepatitis B or patients with cirrhosis (Zhou et al., 2011). **Table 7** summarizes recently described circulating miRNAs that may be used as non-invasive biomarkers for cancer detection. In further examples of the differentiation of diseased patients from healthy controls, circulating miRNAs have also been demonstrated as biomarkers for early-stage cancer diagnosis. For example, Roth et al. noted that the concentration of miR-155 in serum significantly discriminated M0- breast cancer patients from healthy women (Roth et al., 2010). Zhu et al. also identified five miRNAs (miR-16, miR-25, miR-92a, miR-451 and miR-486-5p) as potential markers for the early-stage gastric non-cardia adenocarcinoma (GNCA) (stage I) (Zhu et al., 2014). It has been reported that circulating miR-92a was dysregulated in non-metastatic and metastatic colorectal cancer (CRC) patients and showed potential as a non-invasive biomarker for the early detection of liver metastasis in CRC patients (Wang et al., 2014) .

Circulating miRNA	Types of cancer	Methods	Source
miR-155,-210,-21	Diffuse large B-cell lymphoma	qRT-PCR	serum
miR-132, -181c, -15a, -370, -143-3p, -21-5p, -200a-3p, -646	Cervical squamous cell carcinoma	TaqMan miRNA array, qRT-PCR	serum
miR-122	Chronic hepatitis B	Microarray, qRT-PCR	serum
miR-141	Prostate cancer	qRT-PCR	serum/plasma
miR-1233	Renal cell carcinoma	TaqMan low density array, qRT-PCR	serum
mir-16,-25	Multiple myeloma	NanoString-nCounter microRNA assays	serum
miR-17, -20a, -29c, -223	Nasopharyngeal carcinoma	Microarray, qRT-PCR	serum
miR-17-3p, -29a, -92a, -221	Colorectal Cancer	qRT-PCR	serum
miR-155	Breast Cancer	TaqMan miRNA array, qRT-PCR	serum
miR-21, -26a, -27a, -122, -192, -223, -801	Hepatocellular cancer	Microarray, qRT-PCR	serum
miR-16, -25, -92a, -451, miR-486-5p	Gastric non-cardia adenocarcinoma	TaqMan low density array, qRT-PCR	serum
miR-425-5p, -93-5p	Head and neck cancer	qRT-PCR	Plasma
miR-122	Chronic hepatitis C	qRT-PCR	serum

Tab.7: Summary of the circulating miRNAs that may be used as non-invasive biomarkers for the detection of cancer.

Finally, in a study conducted by Zhi et al., 6 miRNAs, miR-10a-5p, miR-93-5p, miR-129-5p, miR-155-5p, miR- 181b-5p and miR-320d, were found to have significantly different expression levels in AML compared with control serum samples. Furthermore, unsupervised clustering analysis revealed the remarkable ability of the 6-miRNA profile to differentiate between AML patients and normal controls. More importantly, miR-181b-5p levels in serum were significantly associated with overall survival. These data demonstrated that the expression patterns of circulating miRNAs were systematically altered also in AML and miR-181b-5p may serve as a predictor for overall survival in AML patients (Zhi et al., 2013).

2.4.8 MIRNAS IN HEMATOLOGICAL DISEASE.

The general importance of miRNA dysregulation to the pathogenesis of myeloid disorders is implied by the fact that more than 70% of all human miRNAs are encoded within regions of recurrent copy-number alterations in myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) cell lines (Starczynowski et al., 2011). Furthermore, a functional role in the pathology of these diseases has been demonstrated recently by several in vivo mouse models. For example, targeted deletion of *Dicer1* in osteoprogenitor cells resulted in abnormal hematopoiesis, MDS and eventual AML (Raaijmakers et al., 2010). Deletion of murine HSC-encoded *miR-145* and *miR-146a*, miRNAs encoded on the 5q locus, a frequently deleted region in myeloid disorders, caused mild neutropenia, megakaryocytic dysplasia and eventual fatal myeloid malignancy (Starczynowski et al., 2010). Over-expression of *miR-125b* resulted in the development of leukemia, (Bousquet et al., 2010) mirroring what occurs in MDS and AML patients that harbor the t(2,11) translocation involving the encoding locus for this miRNA (Bousquet et al., 2008). Finally, over-expression of *miR-155* (O' Connell et al., 2008) or *miR-29a* (Han et al., 2010) in mouse HSC results in myeloproliferative disorders that can progress to AML.

Many studies have demonstrated that miRNAs are abnormally expressed in myeloid malignancies compared to counterpart controls; most commonly in AML (Marcucci et al., 2011), but also in MDS (Rhyasen et al., 2012) and chronic myeloid leukemia (CML). However it should be noted that the reliability of much of this data is still contentious as the miRNA signatures show little overlap between studies. These discrepancies are probably due to the use of different control populations (unsorted PMBCs, BM-derived $CD34^+$ or in vitro expanded $CD34^+$), as well as technical variability (e.g. differing array platforms, statistical analyses and varying cytogenetic/molecular profiling techniques) between studies. Consequently, the need to validate/standardize these data is a prerequisite to translate these findings into routine clinical practice.

Of particular interest, profiling studies have revealed marked differences in miRNA expression between common cytogenetic subtypes of AML including those harboring favorable-risk abnormalities such as t(8,21), (Jongen et al., 2008; Li et al., 2008; Dixon et al., 2008; Cammarata et al., 2010) inv(16), t(15,17), (Jongen et al., 2008; Li et al., 2008; Dixon et al., 2008) and inv(16), and those with less favorable-risk subtypes such as t(11q23)/*MLL* (Jongen et al., 2008; Li et al., 2008; Dixon et al., 2008) and trisomy 8 cases (Garzon et al., 2008). Additionally, karyotype normal AML patients (i.e. CN-AML) also have distinctive miRNA expression patterns associated with recurrent molecular abnormalities including *FLT3-ITD* (Jongen et al., 2008; Cammarata et al.,2010; Garzon et al., 2008) and *MLL* duplications, mutations in *WT1*,*IDH1*, *IDH2*, *NPM1* (Jongen et al., 2008; Garzon et al., 2008) and *CEPBA* (Jongen et al., 2008) as well as high expression of *BAALC* (Langer et al., 2008) *ERG* and *MN1* (Langer et al., 2009) genes. In addition to chromosomal alterations causing aberrant miRNA expression, epigenetic dysregulation of miRNA plays an important role in myeloid pathogenesis (and in other hematological malignancies), and many are hypermethylated including *miR-124a*, associated with

regulation of EVI1, CEBPA and CDK6, *miR-193a* (targeting KIT) and *miR-34b* (targeting CREB) (Agirre et al., 2012). Conversely miRNAs can target components of the epigenetic machinery such as DNA methyltransferases (Benetti et al., 2008) and histone deacetylases (Roccaro et al., 2010).

The use of miRNA as novel classifiers of myeloid malignancies has drawn a lot of attention recently. In MDS, for example twelve miRNAs were identified as being differentially expressed between high and low risk MDS patients, although numbers in this study were small (n = 25) (Erdogan et al., 2011). Another study of 52 MDS patients correlated high *miR-150* levels with good cytogenetic-risk groups (Roccaro et al,2010). Microarray analyses of samples from 122 AML patients were used to define signatures associated with cytogenetically favorable-risk groups (Garzon et al., 2008). Importantly, these findings were tested in an independent cohort of 60 AML patients using qRT-PCR and levels of *miR-191* and *miR-199a* were found to be independent predictors of prognosis by multivariate analysis. More recently, high levels of *miR-181a* were correlated with better prognostic outcome for 187 CN-AML patients (Schwind et al., 2010).

Like AML and MDS, miRNAs are also aberrantly expressed in CML tumor cells (Agirre et al., 2008). Interestingly, up-regulation of the *miR-17-92* cluster was found to be associated with chronic phase but not blast crisis CML patients (Venturini et al., 2007). The same study demonstrated that K562 cells treated with either imatinib or anti-BCR-ABL siRNA caused down-regulation of these miRNAs. A miRNA signature was derived that could distinguish between CML patients that were responsive and refractory to imatinib treatment (San Jose et al., 2009). The association of miRNAs with BCL-ABL has been further illustrated by the finding that *miR-203*, an epigenetically silenced miRNA in CML, can regulate BCL-ABL expression (Bueno et al., 2008) Additionally, *miR-138*, down-regulated in CML tumor cells but restored in response to imatinib treatment, was recently shown to act as a tumor suppressor in CML cells thorough targeting of BCR-ABL expression as well as enhancing GATA1 activity (Xu et al., 2013).

2.4.9 MICRORNAS IN MYELOPROLIFERATIVE DISORDERS: STATE OF ART.

In recent studies abnormally expressed microRNAs in $CD34^+$ and blood cells of MPN patients have been identified, some of which have a JAK2-dependent pattern of expression (Guglielmelli et al., 2007). Guglielmelli et al. has published a comparative analysis of miRNA expression profile in granulocytes from PMF patients compared to healthy subjects and PV or ET patients (Guglielmelli et al., 2007). A general down-regulation of miRNAs is observed, as in other tumors, although a minority of miRNAs showed increased expression levels (Guglielmelli et al., 2007). Interestingly, the abnormally increased level of over-expressed miR182- and 183 linearly correlated with *JAK2V617F* allelic burden; finally, miRNA panel analysis pointed to discrete differences in 49 miRNAs between *JAK2* mutated and wild-type patients (Guglielmelli et al., 2007). Bruchova and colleagues performed a gene expression profiling of micro-RNAs (miRNAs) in granulocytes from PV patients (Bruchova et al., 2007) and the miRNA signature observed was clearly different from that of normal granulocytes, and allowed the identification of a number of miRNAs (40 out of a total of 326) whose

abnormal expression was either correlated or independent from the *JAK2*V617F mutation. It is of interest, and probably not unexpected, that at least some of the abnormally regulated miRNAs found in PV had been detected in PMF granulocytes in the study of Guglielmelli et al., and that they were also detected in ET or PMF patients by Bruchova et al. Furthermore, Guglielmelli et al. have obtained considerable evidence for a significant contribution of deranged miR-16-2 expression to the pathogenesis of PV (Guglielmelli et al., 2011). Finally, Bortoluzzi et al. performed short RNA massive sequencing and extensive bioinformatic analysis in the *JAK2*V617F-mutated SET2, a factor-independent megakaryoblastic cell line. Overall, 652 known mature miRNAs were detected, of which 21 were highly expressed, thus being responsible of most of miRNA-mediated gene repression (Bortoluzzi et al., 2012). Overall, these data point to a complex pattern of aberrant miRNA expression that might contribute to the molecular complexity of MPNs through transcriptional regulation, and provide a framework for hypothesizing a direct involvement of aberrant miRNA regulation in MPNs, whose deciphering could be of help in better understanding of the whole pathogenetic picture.

3 AIM OF THE STUDY

The molecular basis of MPN has been appreciated recently with the description of mutations in *JAK2*, *MPL and CALR*, but several aspects of their pathogenesis remain elusive. In particular, the mechanisms and/or the molecules involved in the phenotypic mimicry of these disorders, on the basis of an unique mutational event, are largely unknown. To this end, I have been interested in studying microRNAs, a novel class of short non-protein coding RNAs that regulate gene expression through imperfect base pairing with the 3'UTR of target mRNAs, in patients with PMF. Infact, micro-RNAs have been shown to contribute significantly to oncogenesis in different cellular models, including chronic and acute leukemias.

The main objectives of this study were:

(i) to describe abnormally expressed genes and microRNAs in $CD34^+$ cells, granulocytes and plasma;

(ii) to discover novel miRNAs and moRNAs that are specifically associated with the PMF and the other MPNs;

(iii) to address and functionally characterize the mechanistic correlates of abnormalities of selected miRNA expression and/or sequence in the uncontrolled proliferation of MPN hematopoietic cells;

(iv) to establish a potential therapeutic relevance of targeting those miRNAs.

4 MATERIAL AND METHODS

4.1 SAMPLES COLLECTION AND PREPARATION.

4.1.1 PATIENTS

Patients diagnosed with PMF according to World Health Organization (WHO) and International Working Group for Myelofibrosis Research and Treatment (IWGMRT) criteria, respectively, were recruited for this study from the database at Florence hematology unit. Patients provided informed consent for the use of archival material for mutational analysis, and the study was performed under a Florence University Institutional Review Board–approved protocol in referring institutions. The study was conducted in accordance with the Declaration of Helsinki. Samples had been collected at diagnosis of PMF or no later than 1 year afterward provided the patient had remained free of cytotoxic treatment.

4.1.2 MONONUCLEAR AND GRANULOCYTE CELLS PREPARATION.

In order to carry out molecular testing, it was necessary starting from 20 ml of peripheral blood (or 5-8 ml of bone marrow blood) to obtain samples of mononuclear cells and granulocytes.

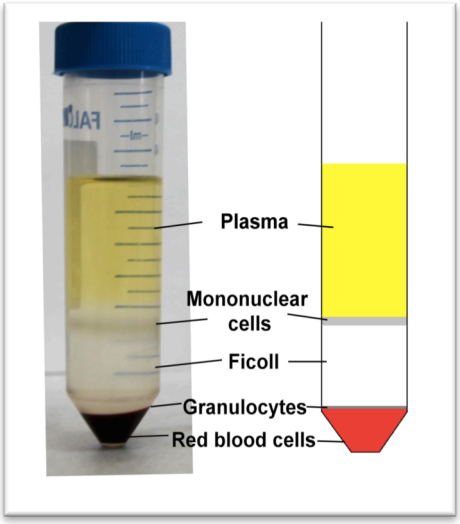


Fig. 13: Separation of human blood cells by Ficoll-Paque

Briefly, whole blood were collected into EDTA-containing polypropylene tubes an processed within 4 hours. Blood was diluted 1:1 with Ca^{2+}/Mg^{2+} free phosphate-buffered saline, carefully layered over 15 ml of Ficoll-Hypaque in a 50-ml tube, and centrifuged at 800 x g at room temperature for 20 minutes **(Fig 13).** Initially it was recovered the ring of mononuclear cells, which were washed in sterile PBS at 4° and centrifuged at 1000 rpm for 10 minutes at 8° C for two times. The cells were counted using a Burker chamber with a dilution factor of 1 : 200.

After, the Ficoll layer were carefully removed and the lower fraction was collected and transferred to a fresh tube. Lysis of red blood cells was performed by the addition of a 10X volume of 1X BD PharmLyse solution (Becton Dickinson BD®), centrifugation of the tube after a 15-minute incubation at room temperature, and removal of the supernatant. This step was repeated twice. After two washes in $Ca^{2+}/Mg2^{+}$ free phosphate-buffered saline, the dry granulocyte pellet was stored at −20°C until processed.

Density gradient–separated mononuclear cells were processed through two sequential steps of immunomagnetic $CD34^{+}$ selection (Miltenyi®); final purity was evaluated by flow cytometry after labeling with PE-HPCA2 anti-CD34 monoclonal antibody (BD Biosciences®), and found to be > 97% in all instances.

4.1.3 CD34$^+$ ISOLATION.

The purification of CD34$^+$ cells was performed starting from mononuclear obtained from peripheral blood or bone marrow aspirates through immunomagnetic separation according to the procedure Mltenyi®**(Fig. 14).** For this procedure it took some reagents such as:

• Buffer 1 (Wash buffer and dilution): Ca^{2+}-Mg^{2+} free PBS, EDTA 2mM;
• Buffer 2 (separation pad): Ca^{2+} -Mg^{2+} free PBS, 0.5% BSA, 2 mM EDTA;
• FcR blocking reagent Human IgG (Miltenyi®);
• MACS CD34 microbeads (Miltenyi®): Microbeads super-paramagnetic conjugated to monoclonal murine anti-human CD34$^+$ (isotype: mouse IgG1).

Once prepared the reagents, the pellet of mononuclear cells was subjected to two washings with buffer 1, centrifuged at 1000 rpm for 10 minutes and then resuspended in buffer 2 to a final volume of 300 µl containing 10^8 mononuclear cells. To the suspension in buffer 2 were then added 100 µl of FcR blocking reagent (Miltenyi®) and 100 µl of CD34$^+$ microbeads (Miltenyi®) for each 10^8 total cells and the whole was incubated for 30 minutes at 4°C and periodically agitated . After incubation buffer 2 was added up to a volume of 10 ml and centrifuged at 1000 rpm for 10 minutes at 8°C; removed very carefully the supernatant, the pellet was resuspended in a final volume of 500 µl of buffer 2 per 10^8 total cells. After washing the column MACS MS$^+$ (Miltenyi®) separation inserted into the magnetic holder with 500 µl of buffer 2, the cells suspension was applied to the column which was then allowed to drain by gravity recovering the fraction CD34$^-$, subsequently they have been performed 3 washes of each column with 500 µl of buffer 2 and they were allowed to flow still collecting them as a fraction CD34$^-$. It was then added a further 500 µl of buffer 2 in the column, which was quickly detached from the magnetic media to recover the fraction of CD34$^+$ cells by means of pressure with a piston-column. In some cases it was necessary to perform additional steps of purification of the CD34$^+$ fraction recovered in a new column up to a maximum of 3 purifications (additional steps do not increase the purity).

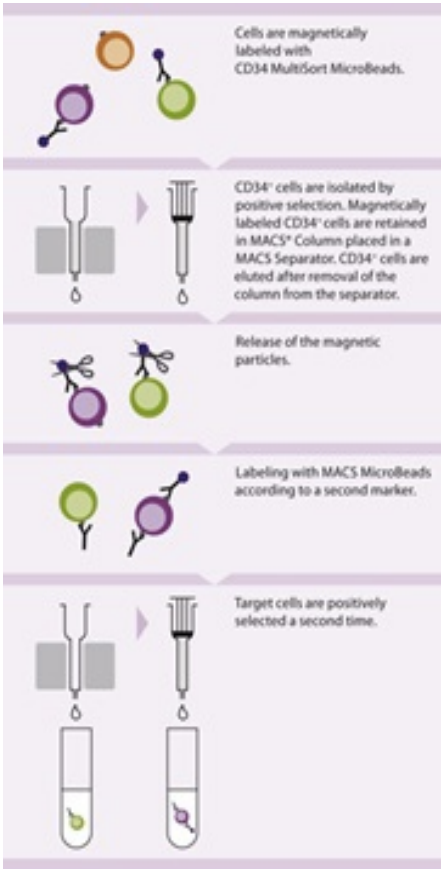


Fig.14: Purification of CD34$^+$ cells procedure.

Purity of the isolated CD34$^+$ cell population was evaluated by flow cytometry after labeling with PE-HPCA2 anti-CD34$^+$ monoclonal antibody (BD Biosciences®) and was always >95%. CD34$^+$ cells (3 *10^5) were immediately lysed in Qiazol (Qiagen®) and were stored at -80°C.

4.1.4 RNA EXTRACTION.

RNA extraction was carried out from the pellet of granulocytes and $CD34^+$ resuspended in Trizol (Invitrogen-Life Technolgies, Inc. ®) (guanidine thiocyanate / phenol that can lyse the cells and inhibit the RNase) following the directions of the company. Each pellet resuspended in Trizol, was incubated at room temperature for 2-3 minutes after adding 0.2 ml of chloroform and centrifuged at 12000 rpm for 15 min at 4°C. They have been obtained so three phases: a upper aqueous phase, an interface and a whitish red phase represented by Trizol. It is the upper aqueous phase was recovered and added to 2 µl of glycogen and 0.5 ml of isopropanol to allow the precipitation of RNA. Following what has been done incubation at room temperature for 10 min with stirring for inversion and centrifugation at 12000 rpm for 10 min at 4°C. At this point it was obtained an RNA pellet which was washed with 75% ethanol and finally centrifuged at 7500 rpm for 5 min at 4°C. The pellet was dried for 5 min and resuspended in 20 µl of water DNAse-free RNAse. Finally, it was gone to quantify and analyze RNA samples obtained.

4.1.5 QUANTIFICATION AND EVALUATION OF THE RNA QUALITY.

RNA obtained was initially quantified by spectrophotometric absorption at 260 nm and it was determined, in the first instance, the value of purity whereas the ratio between the absorbance at 260 nm and that at 280 and 230 nM (Technology Nanodrop®). In order to establish the quality and integrity of each sample of RNA, it has been made an analysis with the Agilent 2100 Bioanalyzer **(Fig.15)**. These technology consists of an actual capillary electrophoresis. Each chip used for this instrument contains a series of tightly interconnected microchannels: the nucleic acid fragments are well separated according to their molecular weight as in a standard electrophoresis in agarose gel. The microchannels of each chip are filled with a matrix and a fluorochrome for which, at the end of the electrophoretic run, the samples are read on the basis of their fluorescence and this information is translated into a typical image of gel electrophoresis and in electropherograms. Specifically, to make the race has been used a chip of type "Nano" (range 50-500 ng of total RNA /uL) and at the end of it has been possible to obtain from the instrument to a defined value RIN (RNA Integrity Number) that allows you to assign a quality index total RNA examined. This parameter has a scale of 10, RNA of excellent quality, in which 0 indicates completely degraded RNA. The RNA samples used showed a RIN greater than 8.

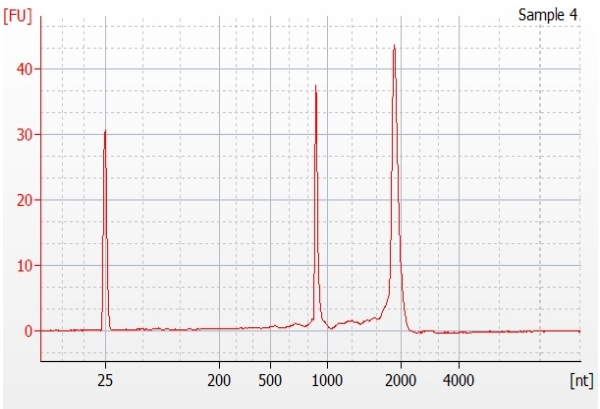


Fig.15: A typical electropherogram of RNA sample obtained by Agilent 2100 bioanalyzer.

4.2 ANALISYS OF GENES AND MIRNAS EXPRESSION PROFILE OF CD34$^+$ CELLS FROM PMF PATIENTS AND CONTROLS.

4.2.1 MICROARRAY DATA ANALYSIS

To obtain gene- and miRNA- expression profiles (GEP and miEP, respectively) CD34$^+$cells were performed on the same RNA isolated from CD34$^+$ cells of 42 PMF and 24 ET patients and 31 healthy donors (n=15 BM, n=16 PB).

With regard to GEP, cDNA synthesis, as well as biotin-labeled target synthesis, was performed using the GeneAtlas 3´ IVT Express Kit (Affymetrix®) according to the standard protocol supplied by Affymetrix. The HG-U219 Array Strip hybridization, staining, and scanning were performed by using the GeneAtlas Platform. To perform miEP, total RNA (100 ng) was labeled using the FlashTag® Biotin HSR kit (Affymetrix®) and hybridized to the Affymetrix Genechip miRNA array 2.0 according to the manufacturer's recommendations. miRNA arrays were processed using the Affymetrix® GeneChip instrument platform. The probe level data were normalized and converted into expression values using the robust multiarray average (RMA) procedure. Quality control assessment was performed using different Bioconductor packages such as R-AffyQC Report, R-Affy-PLM, R-RNA degradation Plot, and QC procedures included in the Partek GS. 6.6 Software Package (http://www.partek.com). Before analysis, a variance filter was applied to remove flat genes with low variation in expression over the samples. An exploratory principal component analysis (PCA) was performed using the PCA module implemented in Partek GS™. Differentially expressed genes (DEGs) and DEMs were then selected following a supervised approach with the analysis of variance (ANOVA) module included in the Partek GS package. In particular, it was selected all the probe sets with a fold change contrast $\geq$2 for DEGs or $\geq$1.5 for DEMs in the pairwise comparison of PMF vs controls and a false discovery rate (q value < 0.05).

4.2.2 VALIDATION GENE EXPRESSION MICROARRAY DATA

To validate the Gene expression array data, at first it was performed a QRT-PCR in an independent cohort of CD34$^+$ cells from 10 PMF patients and 8 healthy subjects (n=4 BM, n=4 PB) and, after, also in an granulocytes of PMF patients (n=32) compared with healthy controls (n= 12).

- Reverse transcription PCR

cDNA was synthesized from total RNA using TaqMan® High capacity cDNA reverse transcription kit (Applied Biosystems®).

The composition of reagents mix for these reverse transcription PCR and a kinetic of reaction are reported below (See Tab. 8 and Tab.9).

Components of reaction	Volume (µL) for one sample
10X RT Buffer	2
25X dNTP Mix (100 mM)	0,8
5X RT Random Primer	2
RNase Inhibitor 20U/µL	1
MultiScribe™ Reverse Transcriptase 50U/µL	1
RNA (~350ng) + H_2O	13,2
TOT	20

Tab. 8: Composition of Retrotrascription PCR mix.

	STEP 1	STEP 2	STEP 3	STEP 4
Temperature	25°C	37°C	85°C	4°C
Duration	10 min	120 min	5 min	∞

Tab. 9 : Reaction kinetics.

- Taqman Real time PCR

To validate the array data in $CD34^+$ cells it was designed a TaqMan low-density array containing 63 + GAPDH TaqMan gene expression assays. The selection of genes was based on either the highest absolute fold-change contrast and/or their putative role in PMF pathogenesis. TaqMan PCR was carried out in duplicate using either Custom TaqMan Array 384-well Cards by using an AB 7900HT Fast Real-Time PCR System (Applied Biosystems®).

The composition of the mix of the reagents which has been used for the process of QRT-PCR and the kinetics of reaction are described respectively in Table 10 and 11.

Components of reaction	Volume (µL) Per Fill reservoir
Taqman Universal PCR Master Mix 2x	50
cDNA sample and RNAse free water	50
TOT	100

Tab. 10: Composition of Taqman® Real time PCR mix for Custom array cards.

STEP	UNG INCUBATION	ENZIME ATTIVATION	PCR cycles (40 cicli)	
			DENATURATION	ANNEALING/EXTENSION
Temperature	50°C	95°C	95°C	60°C
Duration	2 min	10 min	15 sec	1 min

Tab. 11: kinetic of Taqman real time PCR reaction.

- Gene expression in granulocytes samples

After, among the validates genes in previously in $CD34^+$ cells, it was selected a set of 7 genes (See Tab.12) out of those most upregulated to validate their expression in granulocytes.

GENE	PROTEIN NAME	Taqman Assay ID
OLFM4	Olfactomedina 4	Hs00197437_m1
LCN2	Lipocalin 2	Hs01008571_m1
LEPR	Leptin receptor	Hs00174497_m1
FGR	Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog	Hs00178340_m1
ANXA3	Annexin A3	Hs00974395_m1
CEACAM8	Carcinoembryonic antigen-related cell adhesion molecule 8	Hs00266198_m1
DEFA1	Defensin, alpha 1	Hs00234383_m1

Tab. 12: List of selected genes validated in PMF granulocytes.

In these case, each generated cDNA of granulocyte cells was amplified by QRT-PCR with sequence-specific primers from the TaqMan gene expression assays by means of StepOne real-time PCR system (Applied Biosystems®).

The composition of the mix of the reagents which has been used for the process of QRT-PCR is described in Table 13.

Components of reaction	Volume (µL) for one sample
2X Taqman Gene Expression Master Mix	10
20X Gene expression assay	1
20x RNAse P assay	1
cDna	2
H2O	6
TOT	20

Tab. 13: Composition of Taqman® Real time PCR mix.

The kinetics of this reaction is the same as that described previously. Gene expression relative quantification (RQ) was achieved using the comparative cycle threshold (CT) method using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the housekeeping gene. To normalize the data, ΔΔCT was calculated for each sample using the mean of its ΔCT values subtracted from the mean ΔCT value measured in the entire population of healthy subjects, considered as a calibrator; the RQ value was expressed as $2^{-\Delta\Delta CT}$. To make the RQ value symmetric for up- and down-regulated genes, fold change (FC) was used in the Tables for ease of interpretation (for RQ > 1, FC = RQ; for RQ < 1 FC = −1/RQ).

4.2.3 VALIDATION MIRNAS EXPRESSION MICROARRAY DATA.

To validate the miRna expression array data, it was used TaqMan® single miRNA assays to assess the expression of 46 DEMs , which were selected based on either their relatively high differential expression or their biological role in cancer-related processes or myeloid differentiation. TaqMan® assays were carried out in an independent cohort of $CD34^+$ cells from 10 PMF patients and 8 healthy subjects (n=4 BM, n= 4 PB).

For miRNA cDNA synthesis, 800 ng of total RNA was reverse-transcribed using the Taqman® microRNA Reverse Transcription Kit and the miRNA-specific looped-primers included in Megaplex Primer Pool A (Life Technologies®), except for five miRNAs (i.e., miR-1246, miR-378a-3p, miR-543, miR-766-3p, miR-409-3p) out of the above-mentioned pool, which were carried on as individual retro-transcriptions.

The composition of retrotrascription reaction mix is reported in Tab.14 and the thermal cycling conditions in Tab.15.

Components of reaction	Volume for One Sample (µL)
RT Reaction Mix Components	0.8
dNTPs with dTTP (100 mM)	0.2
MultiScribe™ Reverse Transcriptase (50 U/µL)	1.5
10× RT Buffer	0.8
$MgCl_2$ (25 mM)	0.9
RNase Inhibitor (20 U/µL)	0.1
Nuclease-free water	0.2
RNA (~800 ng) + H_2O	3
Total	7.5

Tab. 14: Megaplex RT Reaction Mix Components.

	40 CYCLES			STEP 3	STEP 4
	STEP 1	STEP 2	STEP 3		
Temperature	16°C	42°C	50°C	85°C	4°C
Time	2 min	1 min	1 sec	5 min	∞

Tab. 15: Megaplex RT Reaction Thermal-cycling conditions.

qRT-PCR was performed using TaqMan ®MicroRNA array (Life Technologies®) and the composition of reaction mix is reported below (Table 16).

Components of reaction	Volume (µL) Per Fill reservoir
Taqman Universal PCR Master Mix 2x	50
Megaplex™ RT product and RNAse free water	50
TOT	100

Tab. 16: TaqMan® MicroRNA Array reaction mix.

The thermal cycling condition for Taqman® microRNA real time PCR are the same as described above (see Tab. 11.) and U6 snRNA was used as a housekeeping control.

- miRna expression in granulocytes samples

Among the validated miRNAs in PMF $CD34^+$ cells, it was selected the 24 (Tab 19) most upregulated ones to test their expression in the granulocytes from the same PMF patients (n=30) and healthy donors (n =8) previously used for mRNA expression validation.

Even this time, cDNA was synthesized from total RNA using TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems®) but the composition of the reagents mix and a kinetics of reaction are different than the previous reverse transcription (see Tab.17 and 18).

Components of reaction	Volume (µL) for one sample
10X RT Buffer	**1.5**
25X dNTP Mix (100 mM)	**0.15**
5X RT miRna Primer	**3**
RNase Inhibitor 20U/µL	**0.19**
MultiScribe™ Reverse Transcriptase 50U/µL	**1**
RNA (~10ng) + H_2O	**9.16**
TOT	**15**

Tab. 17 : Composition of a miRna retrotrascription PCR mix.

	STEP 1	STEP 2	STEP 3	STEP 4
Temperature	16°C	42°C	85°C	4°C
Duration	30 min	120 min	5 min	∞

Tab.18: Reaction Kinetics.

Each generated cDNA was amplified by QRT-PCR with sequence-specific primers from the TaqMan®microRNA Assays on Step one plus PCR system (Applied Biosystems®).

The composition of the mix of the reagents which has been used for the process of QRT-PCR and the kinetics of reaction are described respectively in Table 20 and 21 below.

miRna	Location	Sequence
Hsa-miR-127-3p	14q32.2	cugaagcucagagggcucugau
Hsa-miR-146b-5p	10q24.32	ugagaacugaauuccauaggcu
Hsa-miR-152-3p	17q21.32	ucagugcaugacagaacuugg
Hsa-miR-199a-3p	19p13.2	acaguagucugcacauugguua
Hsa-miR-193a-5p	17q11.2	ugggucuuugcgggcgagauga
Hsa-miR-195-5p	17p13.1	uagcagcacagaaauauuggc
Hsa-miR-19a3p	13q31.3	ugugcaaaucuaugcaaaacug
Hsa-miR-19b-3p	13q31.3	ugugcaaauccaugcaaaacuga
Hsa-miR-200c-3p	12q13.31	uaauacugccggguaaugaugga
Hsa-miR-21-5p	17q23.1	uagcuuaucagacugauguuga
Hsa-miR-29a-3p	7q32.3	acugauuucuuuugguguucag
Hsa-miR-335-5p	7q32.2	ucaagagcaauaacgaaaaaugu
Hsa-miR-376c	14q32.31	aacauagaggaaauuccacgu
Hsa-miR-382-5p	14q32.21	gaaguuguucgugguggauucg
Hsa-miR-433	14q32.2	aucaugaugggcuccucggugu
Hsa-miR-485-5p	14q32.31	agaggcuggccgugaugaauuc
Hsa-miR-486-3p	8p11.21	uccuguacugagcugccccgag
Hsa-miR-487b	14q32.31	aaucguacagggucauccacuu
Hsa-miR-494	14q32.31	ugaaacauacacgggaaaccuc
Hsa-miR-660-5p	Xp11.23	uacccauugcauaucggaguug
Hsa-miR-134	14q32.31	ugugacugguugaccagagggg
Hsa-miR-379-5p	14q32.31	ugguagacuauggaacguagg
Hsa-miR-543	14q32.31	aaacauucgcggugcacuucuu
Hsa-miR-409-3p	14q32.31	agguuacccgagcaacuuugcau

Tab. 19: miRnas selected for the validation.

Components of reaction	Volume (µL) for one sample
2X Taqman Universal Master mix, No AmpErase UNG	8
20X miRNA assay	0,8
cDNA	2
H2O	5,2
TOT	16

Tab. 20: Composition of a QRT-PCR mix.

STEP	UNG INCUBATION	ENZIME ATTIVATION	PCR cycles (40 cicli)	
			DENATURATION	ANNEALING/EXTENSION
Temperature	50°C	95°C	95°C	60°C
Duration	2 min	10 min	15 sec	1 min

Tab. 21: Reaction kinetics.

Relative quantities were calculated by using the control group. Quantification of relative miRNA expression on the validation set was performed with the comparative CT method using the formula 2^{-DDCT}, where DDCT = [(CT miRna of interest-CT reference snRNA) PMF sample – mean of (CT miRna of interest-CT reference snRna) control goup] by using snRNAU6 as the reference miRNA .

4.2.4 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA).

Serum levels of LCN2 (NGAL) and OLFM4 secreted proteins in PMF patients and healthy donors were evaluated by ELISA using two commercial kits (NGAL rapid ELISA kit; BioPorto Diagnostics®; Gentofte, and OLFM4 ELISA kit; Antibodies-online GmbH®), according to the manufacturer's instructions. The results are expressed as mean concentration (ng/mL) with each sample assayed in triplicate.

4.2.5 $CD34^+$ CELL-CULTURE CONDITIONS AND ELECTROPORATION.

After immunomagnetic separation, $CD34^+$ cells were seeded in 24-well plates at $5*10^5$/mL in Iscove's-modified Dulbecco medium (IMDM; Euroclone) containing 20% human serum (Bio-Whittaker), stem cell factor (SCF; 50 ng/mL), Fms-like tyrosine kinase 3 ligand (Flt3L; 50 ng/mL), thrombopoietin (TPO; 20 ng/mL), interleukin-6 (IL-6; 10 ng/mL), and interleukin-3 (IL-3; 10 ng/mL; all from Miltenyi).

The electroporation program of $CD34^+$ cells was based on a previously published protocol (Salati et al., 2008) which was optimized to be performed on the 4D-Nucleofector System (Lonza®). Briefly, each sample was electroporated 3 times once every 24 hours with a mix of 3 Silencer Select small interfering RNAs (siRNAs) targeting human *JARID2* (see Tab.22) (Life Technologies®), starting from the day after $CD34^+$ cell purification.

RefSeq Accession number	Gene Symbol	Full Gene Name	Gene ID	siRNA ID	Sense siRNA Sequence	Antisense siRna Sequence
NM_004973	JARID2	jumonji, AT rich interactive domain 2	3720	s7657	GGUUUCUAAG GUAAACGGAtt	UCCGUUUACCU UAGAAACCtg
NM_004973	JARID2	jumonji, AT rich interactive domain 2	3720	s7655	GAAGAACGGG UGGUACGUAtt	UACGUACCACC CGUUCUUCtg
NM_004973	JARID2	jumonji, AT rich interactive domain 2	3720	s7656	GGUGGUACAA GAGAACGAAtt	UUCGUUCUCU UGUACCACCat

Tab. 22: Silencer select small interfering RNAs (siRNAs) targeting human JARID2.

For each electroporation, $4*10^5$ $CD34^+$ cells were resuspended in 100 mL of P3 Primary Cell Solution (Lonza®), containing 3 mg of siRNA mix, and pulsed with the program DS112. To exclude nonspecific effects caused by interfering RNA (RNAi) nucleofection, a sample transfected with a nontargeting siRNA negative control (NegCTR; Silencer Select Negative Control #2 siRNA; Life Technologies®) was included. As described for siRNA transfections, the number of nucleofections and the quantities of miRNA mimics/inhibitors were modified from a previously described protocol (Tenedini et al., 2010) to best fit the properties of the second-generation of miRNA mimics/inhibitors (Life Technologies®). Briefly, $CD34^+$ cells were nucleofected twice, once every 24 hours, with 3 mg of mirVana miR-155-5p mimic or mirVana miRNA mimic Negative Control #1 (Neg-mimic), by using the previously mentioned electroporation protocol DS112. PMF and CB $CD34^+$ cells were nucleofected 4 times, once every 24 hours, with 3 mg of mirVana miR-155-5p inhibitor or mirVana miRNA inhibitor Negative Control #1 (NegINH), by using the electroporation protocol DS112. Cells were analyzed 24 and 48 hours after the last nucleofection for both cell viability and JARID2 or miR-155-5p expression.

After each transfection, $CD34^+$ cells were transferred into pre-warmed fresh medium in 24-well plates (Euroclone®) and maintained in the same culture conditions as described above. For liquid culture differentiation assays, $CD34^+$ cells were plated ($5*10^5$/mL) in IMDM with the addition of 20% BIT 9500 serum substitute (bovine serum albumin, insulin, and transferrin; StemCell Technologies) 24 h after the last nucleofection, in order to set up a multilineage cell culture (SCF, 50 ng/mL; Flt3L, 50 ng/mL; TPO, 20 ng/mL; IL-3, 10 ng/mL; IL-6, 10 ng/mL; all cytokines from Miltenyi ®) and megakaryocyte (MK) unilineage culture(TPO, 100 ng/mL) (Guglielmelli et al., 2009).

4.2.6 METHYLCELLULOSE AND COLLAGEN CLONOGENIC ASSAYS.

The methylcellulose assay was carried out by plating $CD34^+$ cells in MethoCultTM GF H4434 (StemCell Technologies Inc.®), as previously described (Zini et al.,2012). MK colony forming units (CFU-MK) were assayed in collagen-based medium, using a commercial MK assay detection kit (MegaCult-C; StemCell Technologies Inc.®) as previously reported (Zini et al., 2012).

4.2.7 MORPHOLOGICAL AND IMMUNOPHENOTYPIC ANALYSIS.

Differentiation of $CD34^+$ cells was monitored by morphological analysis of May–Grunwald–Giemsa-stained cytospins and by flow cytometric analysis of CD34, CD14, CD66b, CD163, Glycophorin A (GPA), and CD41 surface antigen expression at day 0, 3,5, 8, 10, and 12 after the last nucleofection. Images were captured by using an Ax10 Scope.A1 microscope equipped with an AxioCam ERc 5S Digital Camera and Axion software 4.8 (all Carl Zeiss MicroImaging Inc.®). The images were then processed with Adobe Photoshop 7.0 software. The following monoclonal antibodies (MoAbs) were used for flow cytometric analysis: phycoerythrin (PE)-conjugated mouse anti-human CD14 MoAb, fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD34 MoAb, FITC-conjugated mouse anti-human CD66b MoAb (all from Miltenyi Biotech; Auburn, CA, USA), FITC-conjugated mouse antihuman CD41 MoAb, and PE-conjugated mouse anti-human GPA MoAb (all from Dako®). After staining, cells were analyzed by using a BD FACSCan to II (BD Biosciences ®). At least 10,000 events were counted for each sample to ensure statistical relevance.

4.2.8 LUCIFERASE REPORTER ASSAYS.

Full-length and empty 3´untranslated region (3´UTR) luciferase reporter constructs were all purchased from Labomics®). Every plasmid contains the firefly luciferase gene upstream of a given 3´UTR, and the Renilla luciferase gene acting as a normalizer gene. Mutant 3'UTR luciferase assays were obtained from the above-mentioned plasmids by changing nucleotides 3-5 in the miRNA seed binding site regions (all from Genecopoeia®). K562 cells were electroporated by means of the Amaxa 4D-Nucleofector™ System, according to the manufacturer's instructions. Briefly, K562 cells were subcultured at a density of $3*10^5$ cells/mL 2 days before nucleofection. Cells were then transiently co-nucleofected with either a miRNA mimic or miRNA mimic negative control (NEG-mimic) at a concentration of 3.6 µM and with either 3´UTR-less luciferase or a full-length 3´UTR construct at a concentration of 200 ng/sample. For each electroporation, 10^6 cells were resuspended in 100 µL of SF Cell line Solution (Lonza®) and pulsed with the program FF120. Firefly and Renilla luciferase activities were measured 48 h after electroporation using the Dual-Luciferase Reporter Assay System (Promega), and luminescence was recorded on a GloMax®-Multi+ Detection System with Intinct™ Software (Promega®), according to the manufacturer's protocol. In order to analyze the data, three levels of normalization were performed: first, firefly luciferase activity was normalized to that of Renilla luciferase as the internal control for nucleofection efficiency; second, the data were normalized to the effect of the miRNA mimic on the 3'UTR-less control reporter; lastly, the luminescence signals were normalized to the effect of the Neg-mimic on the corresponding 3´UTR full-length construct.

4.2.9 RETROVIRAL VECTORS PACKAGING.

The human JARID2 cDNA (NM_004973.3) was synthesized and cloned into retroviral vector LXIΔN 6 by GeneArt service (Life Technologies®). Packaging line for LJARID2IΔN

was generated by transinfection in the ecotropic Phoenix and amphotropic GP+envAm12 cells, as previously described (Grande et al.,1999. Viral titers were assessed by flow cytometry analysis of a truncated version of low-affinity nerve growth factor receptor (ΔLNGFR) expression percentage upon infection of KG1 cells or CB $CD34^+$ cells.

4.2.10 HEMATOPOIETIC CELL TRANSDUCTION AND PURIFICATION.

After 24 h of culture, cells were electroporated as previously described in paragraph 4.2.5. Each sample was electroporated twice, once every 24 h, with mirVana™ miR-155-5p mimic or mirVana™ miRNA mimic Negative Control #1 (Neg-mimic) (Life Technologies®). Transduction of CB $CD34^+$ cells was performed after 12 h from the second nucleofection in 24-wells plates at a density of $5x10^5$/ml in IMDM (Euroclone®) containing 20% HS (Bio-Whittaker), SCF (50 ng/ml), Flt3-ligand (Flt3-l) (50 ng/ml), TPO (20 ng/ml), IL-6 (10 ng/ml) and IL-3 (10 ng/ml) (Miltenyi®) (Salati et al., 2008).

Retroviral transduction was performed by four cycles of infection (12 h each one) with viral supernatant with the addition of polybrene (8 µg/ml), 20% HS and human cytokines (SCF, Flt3-l, TPO, IL-6 and IL-3 as already described in CD34+ cell-culture conditions) in retronectin-coated plates. Untreated 24-wells plates were coated with retronectin (106 µg/cm2) (Takara Bio Inc®) following the protocol supplied by the manufacturer. To achieve an optimal expansion and infection of the primary $CD34^+$ cells, the retronectin coated plates were pre-incubated with retroviral supernatant for 4 h, then $CD34^+$ cells were seeded in 24-wells plate at a density of $3*10^5$/ml (1ml/well) in fresh viral supernatant. After transduction, CB $CD34^+$ cells were maintained in the already described liquid culture conditions for 36 h. Transduced $CD34^+$ cells were subsequently purified by an immunomagnetic procedure (EasySep "Do-It-Yourself" Selection Kit®) by means of an antihuman p75-NGFR mouse monoclonal antibody (BD Biosciences®) and seeded in the liquid culture conditions as reported above. Cells were analyzed after the purification of $NGFR^+$cells for JARID2 expression by QRT-PCR. Purity of the isolated $NGFR^+$ cells was evaluated by flow cytometry after labeling with PE anti-NGFR monoclonal antibody (Miltenyi®) at 48 h post-purification and was always >90%.

4.3 CHARACTERIZATION THE NEW MIRNA AND MORNA EXPRESSION IN CD34^{+} USING MASSIVE SMALL RNA-SEQ.

4.3.1 SMALL RNA-SEQ LIBRARY CONSTRUCTION AND SEQUENCING.

For each sample, a small RNA library was prepared starting from 1 µg total RNA, using the TruSeq Small RNA Sample Preparation Kits and protocols (Illumina®, San Diego, CA, USA, http://www.illumina.com). Library quality was checked using High Sensitivity DNA chip (Agilent Technologies®). The purified cDNA libraries was used for cluster generation on Illumina's Cluster Station and sequenced on an Illumina HiSeq2000 instrument, producing single reads from 49 to 57 bp.

4.3.2 SMALL RNA DATA ANALYSIS.

The data analysis of small RNA seq was conducted in collaboration with the group of University of Padua directed by Prof. Bortoluzzi.

It was performed a differential expression analysis using DESeq R/Bioconductor package. It was considered those short RNAs that had a total expression throughout all samples higher than the median. It was performed a multiple test correction according to the Benjamini Hochberg method (FDR). It was considered a corrected p-value of 0.05 as threshold to identify differentially expressed elements.

4.3.3 VALIDATION OF SRNAS CONSIDERED DIFFERENTIALLY EXPRESSED BY RNAseq.

It was performed individual miRNAs assay by Taqman® quantitative real-time PCR (QRT-PCR) for quantification of abnormally expressed miRNAs in PMF and control granulocytes and in CD34^{+} cells. cDNA was synthesized from total RNA using TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems®).The composition of the mix of the reagents which has been used for the process of reverse transcription and the kinetics of reaction are described previously (See par. 4.2). Each generated cDNA was amplified by QRT-PCR with sequence-specific primers from the TaqMan microRNA Assays on Step one plus PCR system (Applied Biosystems®). Relative quantities were calculated by using the control group. Quantification of relative miRNA expression on the validation set was performed with the comparative CT method using the formula $2^{\wedge -DDCT}$, where DDCT = [(CT gene of interest-CT reference gene) PMF sample – mean of (CT miRna of interest-CT reference miRna) control goup] by using snRNAU6 as the reference miRNA .

4.3.4 TARGET PREDICTION OF VALIDATED sRNAS AND FUNCTIONAL ENRICHMENT.

Gene	Location	TaqMan gene expression assays
AGO1	1p34.3	Hs01084653_m1
AGO3	1p34	Hs01087121_m1
AKR1C1	10p15-p14	Hs04230636_sH
BRCA1	17q21	Hs01556193_m1
CAV1	7q31.1	Hs00971716_m1
FSTL1	3q13.33	Hs00907496_m1
FKBP10	17q21.2	Hs0022557_m1
MECOM	3q26.2	Hs00602795_m1
MEIS1	2p14	Hs01017441_m1
MME	3q25.2	Hs00153510_m1
PTPN4	2q14.2	Hs00267762_m1
RAN	12q24.3	Hs03044733_g1
SYS1	20q13.12	Hs01110991_m1
TCF4	18q21.1	Hs00162613_m1
TIMP3	22q12.3	Hs00165949_m1
TNFSF10	3q26	Hs00921974_m1
TRPS1	8q24.12	Hs00936363_m1
JAKMIP2	5q32	Hs00207662_m1

Tab. 23: Target genes of miRnas whose it was analyzed the expression.

The complexity of miRNA-mRNA interactions causes ambiguity in target prediction results.

It was chose to perform a target prediction using two different programs, miRanda and PITA, which implement orthogonal target prediction strategies. Our choice was determined also by code availability, which allowed us to make custom predictions using as query sequences also isomiRs and moRNA sequences.

It was performed a target prediction using both miRanda 3.3a and PITA executable version 6. It was applied default parameters of miRanda to predict target of selected miRNAs and moRNA sequences since these settings are reported to optimize the dynamic programming miRanda algorithm. It was used default parameters for PITA target prediction too. According to PITA documentation, it was considered a binding site with score </= -10 likely to be functional in endogenous microRNA expression levels. It was performed a hypergeometric test using an in house modified version of the R Category package of Bioconductor, which supports Reactome annotation maps via the reactome.db R package.

4.3.5 QRT-PCR ANALYSIS OF TARGET GENE EXPRESSION.

After identifying the target genes of miRNAs, It was analized the expression of some of them considered most relevant (See Tab. 23) using the RNA extracted from $CD34^+$ cells of PMF patients and crontrols. CDNA was synthesized from total RNA using TaqMan® High capacity cDNA reverse transcription kit (Applied Biosystems®). Subsequently cDNA was amplified by QRT-PCR with sequence-specific primers from the TaqMan gene expression assays by means of StepOne real-time PCR system (Applied Biosystems®). Gene expression profiling was achieved using the RQ method as above, using RNase-P as the housekeeping gene.

4.4 EVALUATION OF PLASMA MIRNAS EXPRESSION PROFILE IN PMF PATIENTS.

4.4.1 PLASMA SAMPLES COLLECTION.

All samples were collected in EDTA vacutainer tubes were centrifuged at 1,800 x g (at temperature 4˚C) for 15 min, and subsequently were centrifuged at 7,000 x g for 5 min to remove a cellular debris. In all processing steps, measures were be taken to prevent lysis of cells to do so may lead to contamination of the samples with RNA from intact cells. Once prepared plasma samples may be stored at -80° C.

4.4.2 RNA EXTRACTION AND PURIFICATION FROM PLASMA SAMPLES.

Total RNA was extracted from 200 µl of plasma using miRNeasy Serum/Plasma Kit (Qiagen®). Because plasma samples contain only small amounts of RNA which means that there is a high risk that a significant proportion of the RNA is lost, during extraction, 1 µg of MS2 carrier RNA (0,8 µg/ µl) (Roche Applied Science®) for 200 µl of plasma was added to the QIAzol Lysis Reagent (Qiagen®) prior to RNA purification. Purified RNA was resuspended in 20 µl of nuclease-free water and stored at -80°C prior to assaying miRNA. The use of a carrier ensures the highest and most consistent yield from plasma samples. A source of MS2 carrier is guaranteed to be free from microRNAs.

4.4.3 MIRNA QUANTIFICATION IN PLASMA BY QRT-PCR.

The concentration of purified RNA from plasma or serum is usually very low and it is difficult to carry out a measurement, then, it was decided to use a fixed volumes of eluted RNA (8 µl) for miRNA expression assays as previously reported (MacLellan et al., 2012). RNA was reverse transcribed using the miRCURY LNA Universal RT miRNA PCR, Polyadenylation and cDNA synthesis kit (Exiqon®). The miRNA detection experiments were performed on Serum/Plasma Focus micro-RNA polymerase chain reaction(PCR) Panel (V1) by using SYBR Green master mix (Exiqon®) as recommended by the manufacturer. This panel covers the analysis of 168 human miRNAs.

All qRT-PCR reactions were carried out on 384-well plates in the presence of ROX Reference Dye (Life Technologies®) and analyzed with the Applied Biosystems 7900HT Fast Real-Time PCR System (Life Technologies®). Relative quantities were calculated by using the control group. Quantification of relative miRNA expression on the validation set was performed with the comparative CT method using the formula 2^{-DDCT}, where DDCT = [(CT miRna of interest-CT reference miRna) PMF sample – mean of (CT miRna of interest-CT reference miRna) control goup] by using hsa-miR-93-5p as the reference miRNA .

4.4.4 STATISTICAL ANALYSIS.

Unpaired two-tailed T-test of independent samples as well as one-way analysis of variance (ANOVA) was performed, comparing PMF patients and control groups. GraphPad software (San Diego®) was employed for t-test statistical analysis.

In the discovery set, candidate miRNAs were selected if they were found to be

significantly different expressed in the PMF patients versus control group. Exclusively miRNAs that were found to be up-or down-regulated in each individual PMF patient were selected or the validation set analysis. Statistical significance was defined as P value ≤ 0.05.

4.5 MIRNA EXPRESSION PROFILE IN *JAK2V617F KI* MOUSE MODEL.

4.5.*1* *JAK2V617F KI* MOUSE MODEL.

The analysis of miRNA expression profile was performed on cells purified from *JAK2V617F* KI animals kindly concessed Dr. Jean Luc Villeval (Mouse Clinical Institute, Illkirch, France) where the model was generated. The animal model was developed as follows: a fragment encompassing JAK2 exon 13 was amplified by polymerase chain reaction (PCR) on 129S2/SvPas mouse embryonic stem (ES) cell genomic DNA to introduce the GTC > TTC point mutation (617V > F) and subcloned in a vector with a floxed neomycin resistance (NeoR) cassette resulting in a step 1 plasmid. A 5 homologous arm was amplified by PCR and subcloned in step 1 plasmid to generate a step 2 plasmid. Finally, a 3 homologous arm was subcloned in step 2 plasmid to generate the final targeting construct. Targeted 129S2/SvPas ES clones were confirmed by PCR and Southern blot and injected into C57BL/6J blastocysts to generate chimeric mice. Chimeras (L2) were crossed with flippase (FLP) TG C57BL/6 mice to excise the FRT siteflanked NeoR cassette on F1 progenies (L-). Finally, F1 animals (L-) were crossed with 129Sv mice to generate F2 animals.

4.5.2 ISOLATION OF TER119^{+} AND GR1^{+} CELLS.

Mice belonging to L- progenies (JAK2^{wt}mice) (n=2) and L2 progenies (JAK2^{V617F} mice) (n=2) were sacrified and then bone marrow cells were purified from both femour and tibia. Bones were transfered to 50 mL Falcon tube containing sterile PBS. Immediately after, it was removed a bone from the PBS with tweezers and cut the ends. It was flushed out the bone marrow into the 50 mL Falcon tube by inserting a 20 mL syringe filled with PBS at the knee side of both types of bone. After passing the PBS through the bone, the colour of the bone turned from red to white, indicating that all the marrow was expelled. When all bones were washed of marrow, it was centrifuged cells at 1500 rpm for 5 minutes. TER119^{+} and GR1^{+} cells were obtained by immunomagnetic separation using anti-TER119-mouse and anti-GR1-mouse antibodies (Milteny Biotec®). In adult mice, Anti-TER-119 reacts with 20–25% of bone marrow cells and approximately 50% of spleen cells, but not with thymocytes or lymphonode cells. The mouse Anti-GR-1 antibody reacts with Ly-6G, a 21-25 kDa, GPI-anchored cell surface protein, previously defined as the granulocyte-differentiation antigen-1 (GR-1). GR-1 is expressed on mature granulocytes in bone marrow and peripheral tissues. The Anti-GR-1 antibody also stains monocytes transiently during their differentiation in bone marrow and at low levels plasmacytoid dendritic cells in lymphoid tissues. The quality

of purified TER119^{+} and GR1^{+} populations was evaluated by flow citometer.

4.5.3 RNA EXTRACTION AND MIRNA QUANTIFICATION BY QRT-PCR.

Total RNA was prepared with a Tripure isolation reagent **(**Roche®) as described by the manufacturer. It was added 20 mg Glycogen (Roche ®) to each sample as a carrier.
RNA purity and concentration were evaluated by spectrophotometry using NanoDrop ND-1000. Quality and related size of total and small RNA was assessed by the Agilent 2100 Bioanalyzer microfluidics-based platform (Agilent Technoogies®) with Agilent Small Rna Kit for low molecular weight RNA. Electropherograms were visualized using the Agilent 2100 Expert software wich included data collection, peak detection and interpretation of the different profile (See par 4.1.5). RNA was reverse transcribed using the miRCURY LNA Universal RT miRNA PCR, Polyadenylation and cDNA synthesis kit (Exiqon®) and the analysis of miRNA was performed using that includes miRCURY LNA^{TM} Universal RT microRNA PCR, Ready-to –use Mouse and Rat Panel I (Exiqon®). All qRT-PCR reactions were carried out on 384-well plates in the presence of ROX Reference Dye and analyzed with the Applied Biosystems 7900HT Fast Real-Time PCR System (Life Technologies®).
Relative quantities were calculated by using the L2 and L- mice cells. Quantification of relative miRNA expression on the validation set was performed with th e comparative CT method using the formula 2^$^{-DDCT}$, where DDCT = [(CT gene of interest-CT reference gene) L2 GR1/TER119 – mean (CT gene of interest-CT reference gene) L- GR1/TER119] . The reference miRNA used was been U6 snRNA.

4.5.4 VALIDATION RESULTS DEREGULATED MIRNAS.

To validate results deregulated miRnas, RNA extracted from L2 (N=6) and L- (N=6) GR1/TER119 cells was reverse transcribed using miRna reverse transcription kit (Lifetech®) and a Real-time PCR was performed by TaqMan® miRna assays as described by the manufacturer. All qRT-PCR reactions were analyzed with the Step-one Plus Real-Time PCR System (Life Technologies®). Quantification of relative miRNA expression on the validation list was performed with the comparative CT method as previously described.

4.5.5 STATISTICAL ANALYSIS.

Unpaired two-tailed T-test of independent samples as well as one-way analysis of variance (ANOVA) was performed, comparing miRnas expression of L2 mouse GR1^{+} or TER119^{+} versus a control groups consisting of L- mouse corrispondent cells. In the discovery set, candidate miRNAs were selected if they were found to be significantly different expressed in the L2 mouse cells versus L- control group. Subsequently, a group of miRNAs that were found to be up-or down-regulated in L2 mouse cells at first analisys, were further validated.

5 RESULTS

5.1 ANALISYS OF GENE AND MIRNA EXPRESSION PROFILE OF CD34$^+$ CELLS AND GRANULOCYTES FROM PMF PATIENTS AND CONTROLS.

Norfo R, Zini R, Pennucci V, Bianchi E, Salati S, Guglielmelli P, Bogani C, Fanelli T, ***Mannarelli C*** *et al. (2014) Blood. 124(13):e21-32.*

5.1.1 GENE EXPRESSION PROFILE OF CD34$^+$ CELLS FROM PMF PATIENTS.

It was performed mRNA expression profiling in CD34$^+$ cells from 42 PMF patients (n= 23 *JAK2V617F*-positive and n=19 wild-type JAK2) and 31 healthy donors (n=15 from the BM and n=16 from the PB). After data preprocessing, using an ANOVA-based supervised approach for comparing PMF samples with both BM and PB controls, it was identified 718 DEGs. Array data confirmed the abnormal expression of several genes (ie, WT1, NFE2, CXCR4, and CD9) previously identified as deregulated in PMF CD34$^+$ cells by our group in a different cohort of PMF patients (Guglielmelli et al, 2007).
Moreover, PMF samples exhibited increased levels of several putative cancer markers, such as ANGPT1, CEACAM8, and CP, previously reported to be associated with poor prognosis in hematologic and solid neoplasms, as well as genes associated with BM fibrosis (LEPR, MMP9, and TIMP3) and aberrant migration (TM4SF1, RHOB, ARHGAP18, and MMP8). In addition, PMF samples showed a deregulated expression pattern of a number of transcription factors and chromatin remodelers involved in myeloid and MK commitment, either downregulated (ie, JARID2, RUNX2, KLF3, and AFF3) or upregulated (ie, FHL2, MAF, and IKZF2). A selected list of DEGs chosen for their biological significance is presented in **Table 24**. To validate the array data, it was designed a TaqMan® low-density array containing 63 and GAPDH TaqMan® gene expression assays. The selection of genes was based on either the highest absolute fold-change contrast and/or their putative role in PMF pathogenesis. TaqMan® assays were carried out in an independent cohort of CD34$^+$ cells from 10 PMF patients and 8 healthy subjects (n=4 BM, n=4 PB) and enabled validation of the expression of 50 out of 63 genes (79.4%).

Gene symbol	Gene ontology function	Notes	Fold change
ANGPT1	Secreted protein	Overexpressed in AML, CML, and MDSs	3.1
ANXA3	Cytoplasmatic protein	Negative prognostic factor for prostate cancer	10.4
ARHGAP18	Cytoplasmatic protein	Involved in cell spreading and motility	3.0
CD9	Membrane protein	Involved in platelet activation and aggregation; involved in BM remodeling in PMF	2.2
CEACAM8	Membrane protein	Overexpressed in imatinib-resistant CML cells	5.5
CP	Enzyme	Overexpressed in AML; negative prognostic factor for renal carcinoma	2.6
DEFA1	Secreted protein	Overexpressed in imatinib-resistant CML cells; biomarker for diagnosis of CRCA	60.2
FGR	Kinase	Involved in cell migration	1.9
FHL2	Transcription factor	Promotes myeloid proliferation; overexpressed in AML	2.7
IDH1	Cytoplasmatic protein	Mutated in MPNs	2.4
IFI27	Membrane protein	Involved in defense and immunity	2.4
IFIH1	Cytoplasmatic protein	Involved in defense and immunity	2.3
IKZF2	Transcription factor	Overexpressed in in Hodgkin lymphoma and ALL	3.6
ITGB3	Membrane protein	Involved in platelet activation and aggregation	2.5
LCN2	Secreted protein	Expression induced by BCR-ABL protein; negative prognostic factor for breast cancer	7.0
LEPR	Membrane protein	Overexpressed in AML and PMF; involved in fibrosis	9.7
MAF	Transcription factor	Negative prognostic factor for MM	8.0
MEF2C	Transcription factor	Involved in MK differentiation	2.3
MMP9	Extracellular matrix protein	Involved in the development of fibrosis	3.8
MYC	Transcription factor	Involved in MK differentiation; cancer marker	2.8
NFE2	Transcription factor	Involved in MK differentiation; overexpressed in PMF	2.0
OLFM4	Secreted protein	Negative prognostic factor for colorectal, breast, and lung cancer	3.5
PF4	Secreted protein	Involved in platelet activation and aggregation	3.4
PIM1	Transcription factor	Overexpressed in PMF	2.6
RHOB	Cytoplasmatic protein	Involved in cell spreading and motility	3.7
TIMP3	Extracellular matrix protein	Involved in the development of fibrosis	4.0
TM4SF1	Membrane protein	Involved in cell spreading and motility	4.4
VWF	Secreted protein	Highly expressed by early MKs; involved in platelet adhesion	4.4
WT1	Transcription factor	Negative prognostic factor in AML; associated with high severity score in PMF	2.0
AFF3	Transcription factor	Fusion with MLL gene in ALL and with RUNX1 gene, partner of MLL	−2.2
ARHGEF7	Cytoplasmatic protein	Involved in cell migration, attachment, and cell spreading	−2.2
ARID4A	Nuclear protein	Involved in chromatin remodeling; K/O mice develop myelofibrosis	−2.3
BRWD1	Nuclear protein	Involved in chromatin remodeling	−1.8
CDC42	Cytoplasmatic protein	Cdc42-deficient mice developed a fatal myeloproliferative disorder	−4.9
CEBPD	Transcription factor	Myeloid commitment regulator	−2.2
CEBPG	Transcription factor	Myeloid commitment regulator	−2.5
CXCR4	Membrane protein	Involved in BM homing	−2.5
EIF2AK3	Kinase	The ablation in tumor cells results in accumulation of ROS	−3.0
FOXO1	Transcription factor	Involved in OXS response; negative prognostic factor for AML	−2.3
HMGB3	Nuclear protein	Involved in chromatin remodeling; required for the proper balance between HSC self-renewal and differentiation	−2.5
IRF4	Transcription factor	Downregulated in CML	−2.7
IRF8	Transcription factor	Downregulated in CML	−6.4
JARID2	Nuclear protein	Involved in chromatin remodeling and in AML progression	−2.5
KLF3	Transcription factor	Downregulated in AML; K/O mice display abnormalities in hematopoiesis	−3.6
MAFF	Transcription factor	Myeloid commitment regulator	−2.0
MEF2D	Transcription factor	Involved in myogenic differentiation; fusion with DAZAP1 gene in ALL	−2.7
MLL5	Transcription factor	Frequently deleted in human myeloid malignancies	−2.7
MXD1	Transcription factor	Involved in regulation of cell proliferation; antagonizes MYC gene	−3.4
NR4A3	Nuclear protein	Involved in chromatin remodeling; hypoallelic mice display MDS/MPNs features	−2.4
NUP98	Transcription factor	Involved in fusions with different partner genes in patients with hematopoietic malignancies	−2.5
PHC3	Nuclear protein	Component of polycomb repressive complex	−2.1
PURB	Nuclear protein	Deleted in MDS and AML	−2.5
RUNX2	Transcription factor	Involved in hematopoietic and osteogenic lineages differentiation	−2.1
SF3B1	Nuclear protein	Mutated in MDS and in MDS/MPNs	−2.0
SMAD7	Transcription factor	Involved in fibrosis; downregulated in MDSs	−7.2
TCF4	Transcription factor	Myeloid commitment regulator	−2.4
TLE4	Transcription factor	Deleted in AML	−2.2
TP53INP1	Nuclear protein	Loss of expression in several cancers; inactivation correlates with increased cell migration	−2.5

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; BCR-ABL, breakpoint cluster region gene-Abelson murine leukemia viral oncogene homolog 1; CML, chronic myeloid leukemia; CRCA, colorectal cancer; MDS, myelodysplastic syndromes; MM, multiple myeloma; OXS, oxidative stress; ROS, reactive oxygen species.

Tab.24: DEGs selected by biological significance.

5.1.2 MIRNA EXPRESSION PROFILE OF CD34^{+} CELLS FROM PMF PATIENTS.

To draw a comprehensive picture of miRNA deregulation and its relationship with differential gene expression in PMF cells, it was performed miEP in the same sample set, using the Affymetrix miRNA 2.0 arrays.

Next, by using an ANOVA-based supervised approach as described in paragraph 4.2.1, it was selected 76 DEMs. In particular, it was found several upregulated miRNAs associated with hematologic malignancies, or known as oncomiRs (ie, miR-155-5p, miR-21-5p, miR-29a-3p, and miRNAs belonging to the miR-17-92 cluster) (Schotte et al,

2012). By contrast, among the downregulated miRNAs, it was found miR-378c, which is described as a tumor suppressor gene in gastric cancer (Deng et al., 2013). Furthermore, overexpressed miRNAs previously identified as being involved in MK commitment were also found (ie, miR-146b-5p and miR-34a-5p) (Li et al., 2011). To validate the array data, it was used TaqMan® single miRNA assays to assess the expression of 46 DEMs, which were selected based on either their relatively high differential expression or their biological role in cancer-related processes or myeloid differentiation. TaqMan® assays were carried out in an independent cohort of $CD34^+$ cells from 10 PMF patients and 8 healthy subjects (n=4 BM, n =4 PB) and confirmed the deregulated expression of 34 out of 46 miRNAs (73.9%).

5.1.3 VALIDATION OF A GENE SET ON GRANULOCYTES AND SERUM FROM PMF PATIENTS.

Among the 50 validated genes described previously, it was selected a set of 7 genes (OLFM4, LCN2, LEPR, FGR, ANXA3, CEACAM8, and DEF1A) out of those most upregulated to validate their expression in granulocytes, which represent a more appropriate source for diagnostic/prognostic purposes. Using qRT-PCR, it was observed that OLFM4, LCN2, LEPR, FGR, and ANXA3 mRNA levels were significantly increased in PMF granulocytes (n=32) compared with healthy controls (n= 12) (Fig 16 A), whereas CEACAM8 and DEF1A expression was not statistically modulated between the 2 groups (data not shown). Because OLFM4 and LCN2 genes encode for 2 secreted proteins, it was assessed OLFM4 and LCN2 protein levels in the serum of the same PMF patients (n=32) and healthy donors (n=8) by means ofan ELISA. As shown in Figure 16B, the levels of OLFM4 and LCN2 secreted proteins were significantly higher in PMF patients than in healthy donors. Of note, the median concentration of OLFM4 serum protein was five fold higher in PMF patients than in controls.

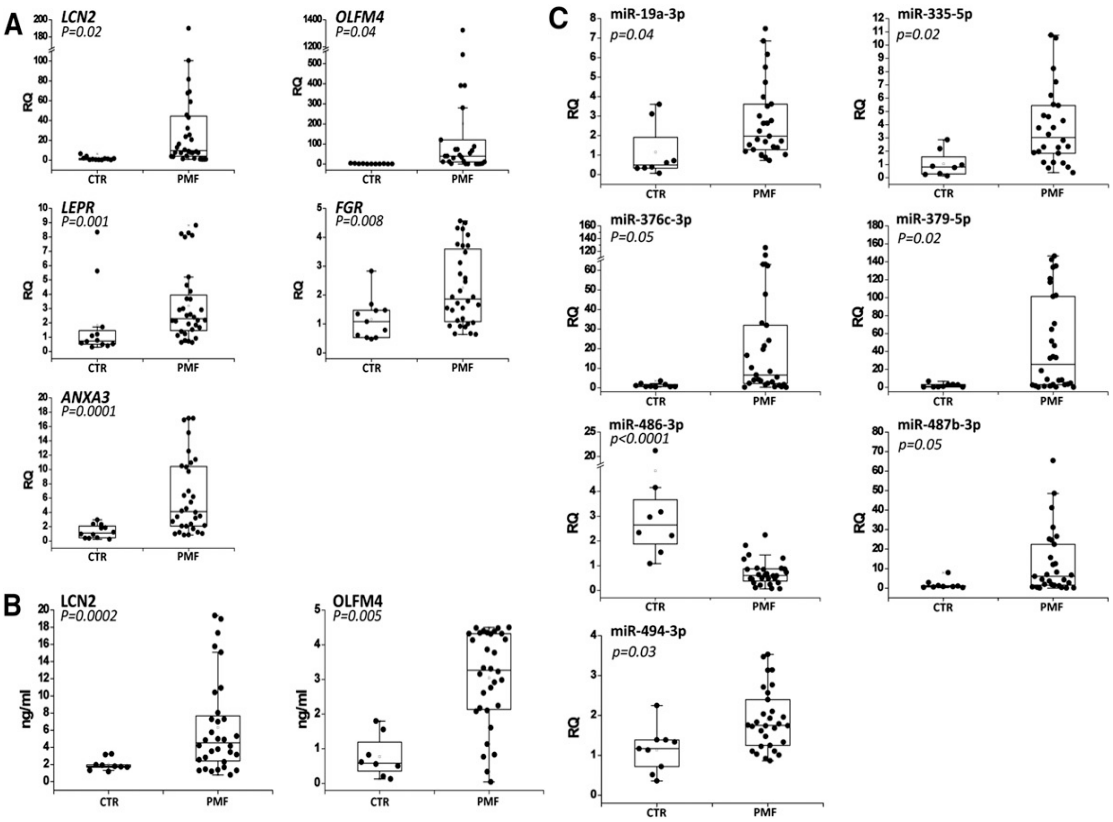


Fig. 16. Validation of the selected genes and miRNAs in granulocytes and plasma from PMF patients. (A) Expression of the 7 selected genes in granulocytes from PMF patients and healthy donors. Gene expression levels were measured by qRT-PCR starting from granulocyte total RNA and were expressed as relative quantity (RQ). Boxes represent the inter quartile range that contains 50% of the subjects, the horizontal line in the box marks the median, and the bars show the range of values. Data are representative of 32 PMF and 12 control (CTR) samples. (B) Serum levels of 2 secreted proteins (LCN2 and OLFM4) in PMF patients and healthy donors. Protein levels were measured by enzyme-linked immunosorbent assay (ELISA) and were expressed as ng/mL. Boxes represent the interquartile range that contains 50% of the subjects, the horizontal line in the box marks the median, and the bars show the range of values. Data are representative of 30 PMF and 8 CTR samples. (C) Expression levels of the 8 selected miRNAs in granulocytes from PMF patients and healthy donors. The miRNA expression levels were measured by qRT-PCR starting from granulocyte total RNA and were expressed as RQ. Boxes represent the interquartile range that contains 50% of the subjects, the horizontal line in the box marks the median, and the bars show the range of values. Data are representative of 32 PMF and 12 CTR samples. *P≤ 0.05 vs CTR.

5.1.4 VALIDATION OF THE SELECTED MIRNAS IN THE GRANULOCYTES FROM PMF PATIENTS.

Among the validated miRNAs in PMF CD34$^+$ cells, it was selected the 16 most upregulated ones (see Table 19 in "Material and methods") to test their expression in the granulocytes from the same PMF patients (n=30) and healthy donors (n=8) previously used for mRNA expression validation. It was demonstrated that the levels of miR-19a-3p, miR-335-5p, miR-379-5p, miR-376c-3p, miR-487b-3p, and miR-494-3p were significantly increased in PMF granulocytes compared with controls; whereas miR-486-3p expression was significantly decreased in PMF granulocytes, in contrast to the results obtained in CD34$^+$cells (Figure 15C). The levels of the remaining miRNAs were not statistically modulated between PMF and controls.

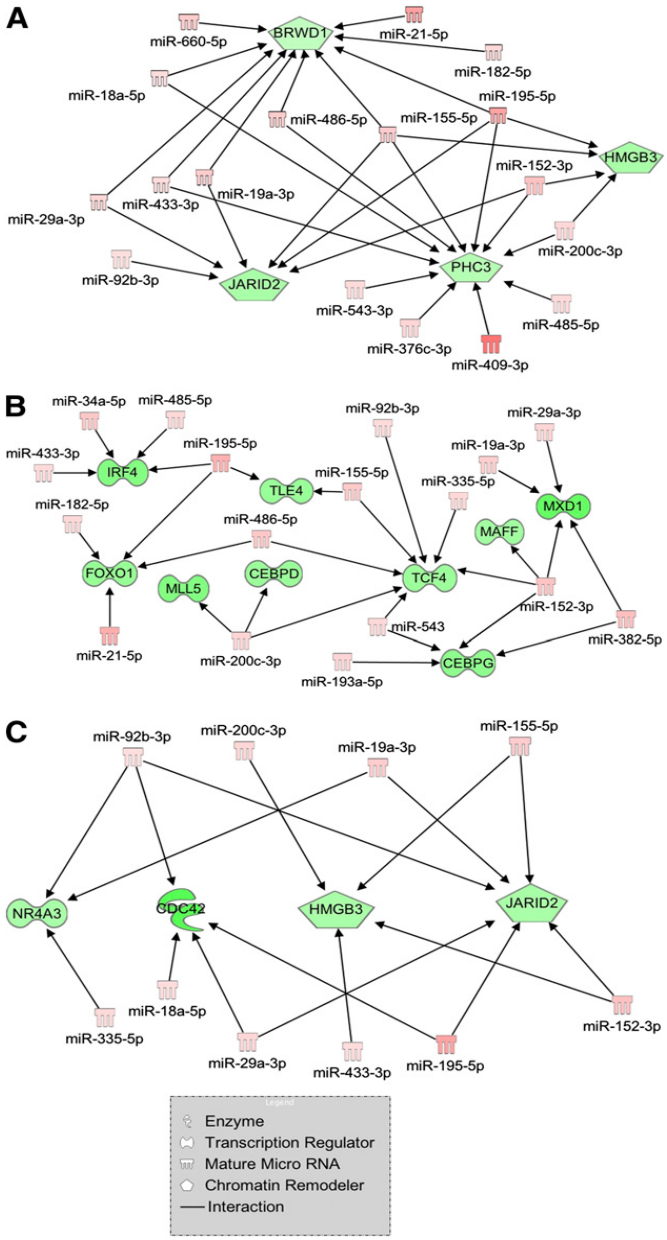


Fig. 17. Regulatory networks of mRNA-miRNA interactions built through IPA.Visualization of the regulatory networks enriched for chromatin remodeling genes (A), myeloid transcription factors (B), and myeloproliferative disorder-related genes (C). Red filling means upregulation, and green filling indicates downregulation

5.1.5 GEP AND MIEP INTEGRATIVE ANALYSIS.

Because each miRNA can target many mRNAs while a single mRNA can be targeted by multiple miRNAs, it was performed IA by means of IPA to untangle this combinatorial complexity. Based on IPA, it was selected DEM-DEG pairs with an anticorrelated expression pattern. In particular, 56 out of the 76 DEMs have at least 1 anticorrelated target among DEGs; whereas 445 out of the 718 DEGs have at least 1 anticorrelated targeting DEM. There were 1167 anticorrelated miRNA-target pairs finally generated. Among the interaction networks uncovered by IPA, it was focused our attention on 3 of them because of their enrichment in genes and miRNAs involved in myeloproliferative disorders and/or in hematopoietic differentiation. Figure 17A shows several miRNAs that are highly expressed in PMF, such as miR-18a-

5p, miR-29a-3p, miR-433-3p, miR-19a-3p, miR-155-5p, miR-195-5p, miR-200c-3p, and miR-152-3p, and their downregulated targets (BRWD1, JARID2, PHC3, and HMGB3) implicated in chromatin remodeling, a process severely impaired in MPNs. The second network (Figure 17B) displays several upregulated miRNAs involved in myeloid differentiation (miR-155-5p, miR-21-5p, and miR-29a-3p) (O'Connelet al., 2011) and their interactions with downregulated transcription factors, known as leukemic tumor suppressors (TLE4, MLL5, and FOXO1) or myeloid commitment regulators (CEBPD, CEBPG, MAFF, TCF4, and MXD1). Finally, through IA we could identify a regulatory network gathering a high number of upregulated oncomiRs (ie, miR-155-5p, miR-29a-3p, miR-92b-3p, miR-19a-3p, and miR-18a-5p) targeting anticorrelated mRNAs whose downregulation or deletion has been related to hematopoietic disorders. In particular, hypoallelic NR4A3 or CDC42 K/O in mice leads to a myeloproliferative disorder (Ramirez-Herrick et al., 2011; Yang et al., 2007) and JARID2 is a frequently deleted gene in leukemic transformation of chronic myeloid malignancies (Puda et al., 2012); the other target, HMGB3, is instead described as a regulator of self-renewal/ differentiation balance in murine HSCs (Nemeth et al., 2006) (Figure 17C).

5.1.6 MIRNA-MRNA INTERACTION VALIDATION BY LUCIFERASE REPORTER ASSAYS.

Because the network shown in Figure 17C contained the highest number of oncomiRs and targets involved in malignant hematopoiesis, in collaboration with the laboratory directed by Prof.ssa R. Manfredini in the University of Modena and Reggio Emilia, it was selected to validate every putative miRNA/target pair by assessing 3'UTR luciferase activity upon miRNA overexpression; K562 cells were selected as an in vitro system providing a hematopoietic background.

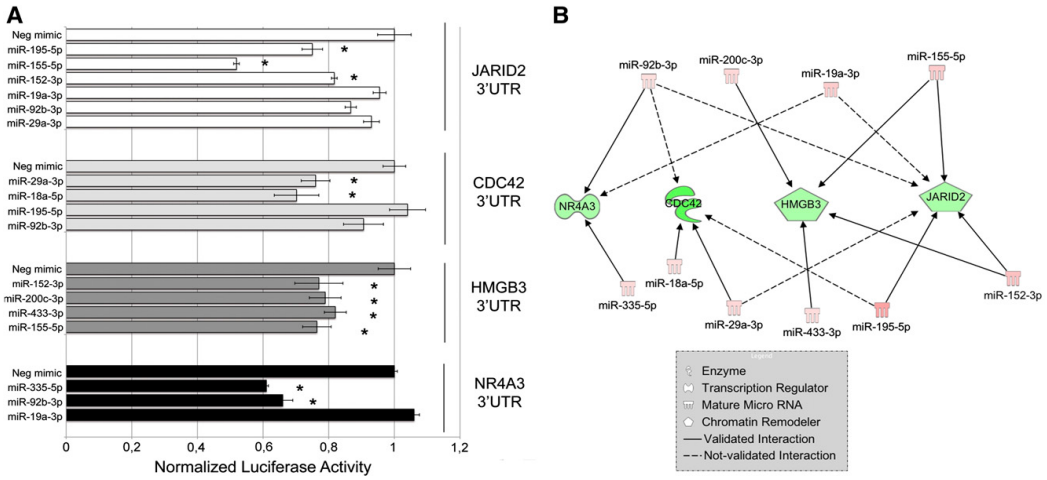


Fig. 18. Validation of 3'UTR-miRNA interactions. (A) Normalized luciferase activity of K562 cells nucleofected with the indicated miRNA mimics and 3'UTR luciferase reporter vectors. Firefly luciferase activity was measured 48 hours after nucleofection and normalized to Renilla luciferase activity. Values are reported as mean 6 SEM; *P<0.05 vs miRNA mimic negative control (Neg-mimic). Results come from 3 independent experiments performed in duplicate. (B) Graphical representation of mRNA-miRNA interactions validated by means of 3'UTR luciferase reporter assays. Solid lines (validated interactions); dashed lines (not validated interactions).

As shown in Figure 18A, the data demonstrated that the following 3'UTR-miRNA interactions were statistically significant: JARID2 3'UTR by miR-152-3p, miR-195-5p,

and miR-155-5p; CDC42 3’ UTR by miR-29a-3p and miR-18a-5p; HMGB3 3’UTR by miR-152-3p, miR-200c-3p, miR-433-3p, andmiR-155-5p; NR4A3 3’UTR by miR-335-5p and miR-92b-3p. Conversely, the interactions between the remaining miRNA-3’ UTR target pairs were not confirmed. Figure 18B graphically recapitulates all the confirmed and non confirmed interactions of the IA network previously shown.

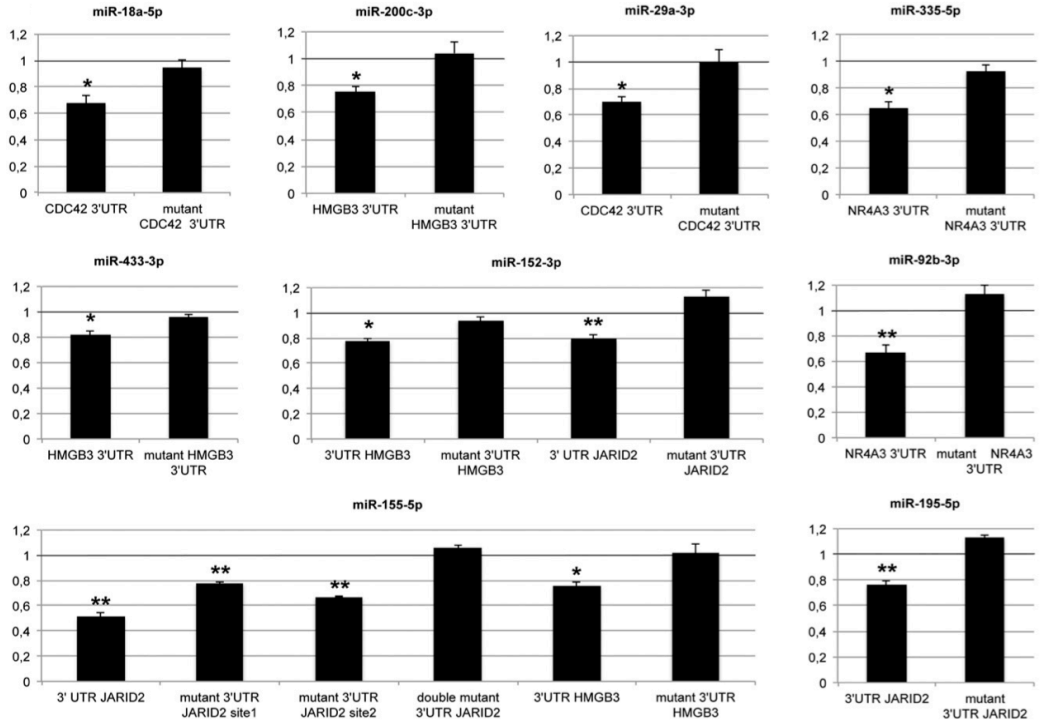


Fig. 19: Results of the luciferase reporter assays performed with wild-type and mutant 3’UTR. Assays were carried out only for the 3’UTR-miRNA interactions previously validated (Fig. 18A). Each bar represents the luciferase activity upon miRNA overexpression normalized on the value of the same 3’UTR luciferase vector upon Neg-mimic transfection. Values are reported as mean 6 SEM; *P< 0.05 vs mutant 3’UTR. Results come from 3 independent experiments performed in duplicate.

Figure 19 clearly shows that mutations in the miRNA binding site of 3’UTR targets prevented the decrease in luciferase reporter activity by miRNAs, which conversely occurs in their wild-type counterparts. Collectively, the luciferase assay data support the good predictive power by IA, as demonstrated by the 11/17 (64.7%) successful predictions for the selected network.

5.1.7 SILENCING OF JARID2 IN NORMAL CD34$^+$ CELLS.

Because the contribution of JARID2 to PMF pathogenesis has never been investigated, it was performed RNAi-mediated gene silencing experiments on normal CD34$^+$ cells. First, it was optimized the CD34$^+$ cell nucleofection protocol for the Amaxa 4D-Nucleofector System technology . CD34$^+$ cells were transfected with a mixture of 3 Silencer Select siRNAs targeting JARID2 mRNA and with a non targeting siRNA as NegCTR. The expression level of JARID2 in control samples and JARID2-siRNA cells was assessed by qRT-PCR at 24 and 48 hours after the last nucleofection (Figure 20A).

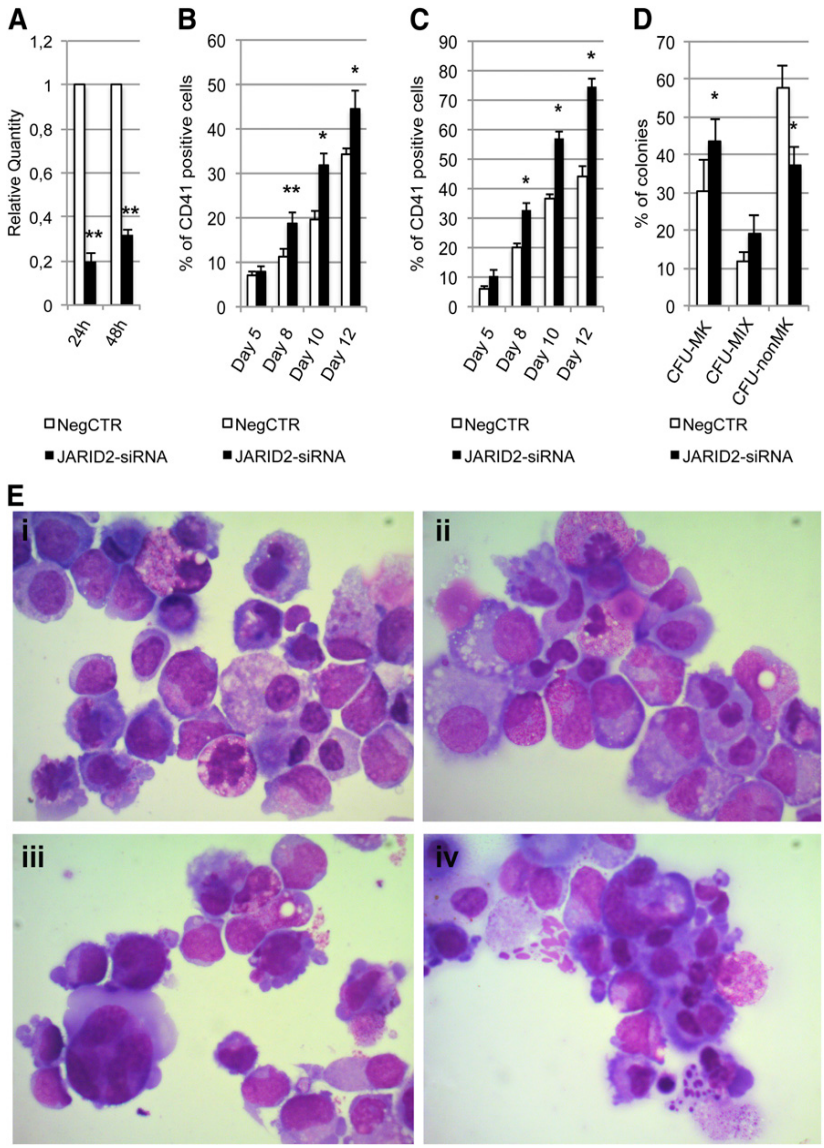


Fig. 20:. Effect of JARID2 silencing on normal CD34$^+$ cell differentiation. (A) Expression levels of JARID2 at 24 and 48 hours after the last nucleofection were measured by qRT-PCR, and data are reported as RQ. (B-C) Results of the statistical analysis on the percentage of CD41$^+$ cells performed by flow cytometry at days 5, 8, 10, and 12 after the last nucleofection on serum-free multilineage and MK unilineage cultures. (D) Results of the statistical analysis of collagen-based clonogenic assay. The cells were plated 24 hours after the last nucleofection and scored after 12 days. (E) Morphologic analysis of NegCTR (i-ii) and JARID2-siRNA (iii-iv) samples after May-Grunwald-Giemsa (MGG) staining at days 8 and 10 of MK unilineage culture after the last nucleofection in a representative experiment. Values are reported as mean 6 SEM. **P<0.01 vs NegCTR; *P<0.05 vs NegCTR. The results come from 5 independent experiments.

Flow cytometric analysis of the CD4$^+$ MK marker performed on serum free multilineage culture at days 8, 10, and 12 showed that JARID2 inhibition induces a significant increase in the MK fraction compared with the NegCTR sample (Figure 20 B). Unilineage MK differentiation culture experiments further confirmed these results (Figure 20C). Flow cytometric analysis of granulocytic, mono-macrophagic, and erythrocyte differentiation markers did not highlight any significant modulation between JARID2-siRNA CD34$^+$ cells and the NegCTR sample (data not

shown). The methylcellulose assay indicated a 1.5-fold increase in the clonogenic efficiency of JARID2-siRNA CD34$^+$ cells vs the NegCTR sample, whereas there was no significant difference in the percentage of erythroid and myeloid colonies (data not shown). Next, it was examined the effect of JARID2 silencing on MK commitment by plating NegCTR and JARID2-siRNA CD34$^+$ cells in a collagen-based serum-free semisolid culture medium that supports the growth of MK progenitors in vitro. The results, reported in Figure 20D, demonstrated that JARID2 silencing induces a remarkable increase in colony forming unit (CFU)-MKs and a strong decrease of non-MK colonies (CFU non-MK) compared with the NegCTR sample. Moreover, morphologic evaluation of MGG-stained cytospins of thrombopoietin-treated cells at days 8 and 10 after the last nucleofection clearly displayed a considerable enrichment in MK precursors at different stages of maturation in JARID2-siRNA cells compared with NegCTR (Figure 20E). Finally, to better characterize the changes in gene expression induced by JARID2 gene silencing, it was performed mRNA profiling in NegCTR and JARID2-siRNA CD34$^+$ cells.

5.1.8 MIR-155-5P OVEREXPRESSION AND SILENCING IN NORMAL AND PMF CD34$^+$ CELLS.

Next, it was asked whether any JARID2-targeting miRNA could reproduce its silencing effects on MK differentiation. Thus, it was decided to overexpress miR-155-5p in normal CD34$^+$ cells because it was highlighted by the luciferase reporter assay as the strongest regulator of the JARID2 3'UTR (Figure 19A). Furthermore, a significant negative correlation was observed between miR-155-5p and JARID2 expression levels across the whole microarray data set. CD34$^+$ cells were transfected either with miR-155-5p mimic or with Neg-mimic. By means of qRT-PCR, it was observed that the JARID2 mRNA level was downregulated upon miR-155-5 overexpression (RQ +/- SEM, 34.7 +/- 11.1, $P< 0.05$) at 24 and 48 hours after the last nucleofection (Figure 20Ai).

As observed for the JARID2 knockdown, miR-155-5p overexpression led to a significant increase of the percentage of CD41$^+$ cells at days 10 and 12 after the last nucleofection in serum-free multilineage culture (Figure 21Aii). Similar results were obtained under unilineage MK differentiation culture conditions (Figure 21Aiii). Furthermore, the collagen-based assay showed that miR-155-5p overexpression causes a significant increase in the CFU-MKpercentage coupled with a strong decrease of non-MK colonies (Figure 21Aiv).

Finally, the morphologic analysis of MGG-stained cytospins showed a remarkable enrichment in MK precursors at different stages of maturation in miR-155-5p overexpressing cells compared with controls at days 10 and 12 after the last nucleofection (Figure 21Av). Furthermore, the study was aimed at assessing whether miR-155-5p downregulation in PMF CD34$^+$ cells could reduce the expansion MK lineage. First, we evaluated JARID2 levels upon miR-155-5p silencing, observing a statistically significant increase at 24 and 48 hours after the last nucleofection (Figure 21Bi). As observed for the JARID2 knockdown, miR-155-5p overexpression led to a

significant increase of the percentage of CD41^{+}cells at days 10 and 12 after the last nucleofection in serum-free multilineage culture (Figure 21Aii). Similar results were obtained under unilineageMK differentiation culture conditions (Figure 20Aiii).

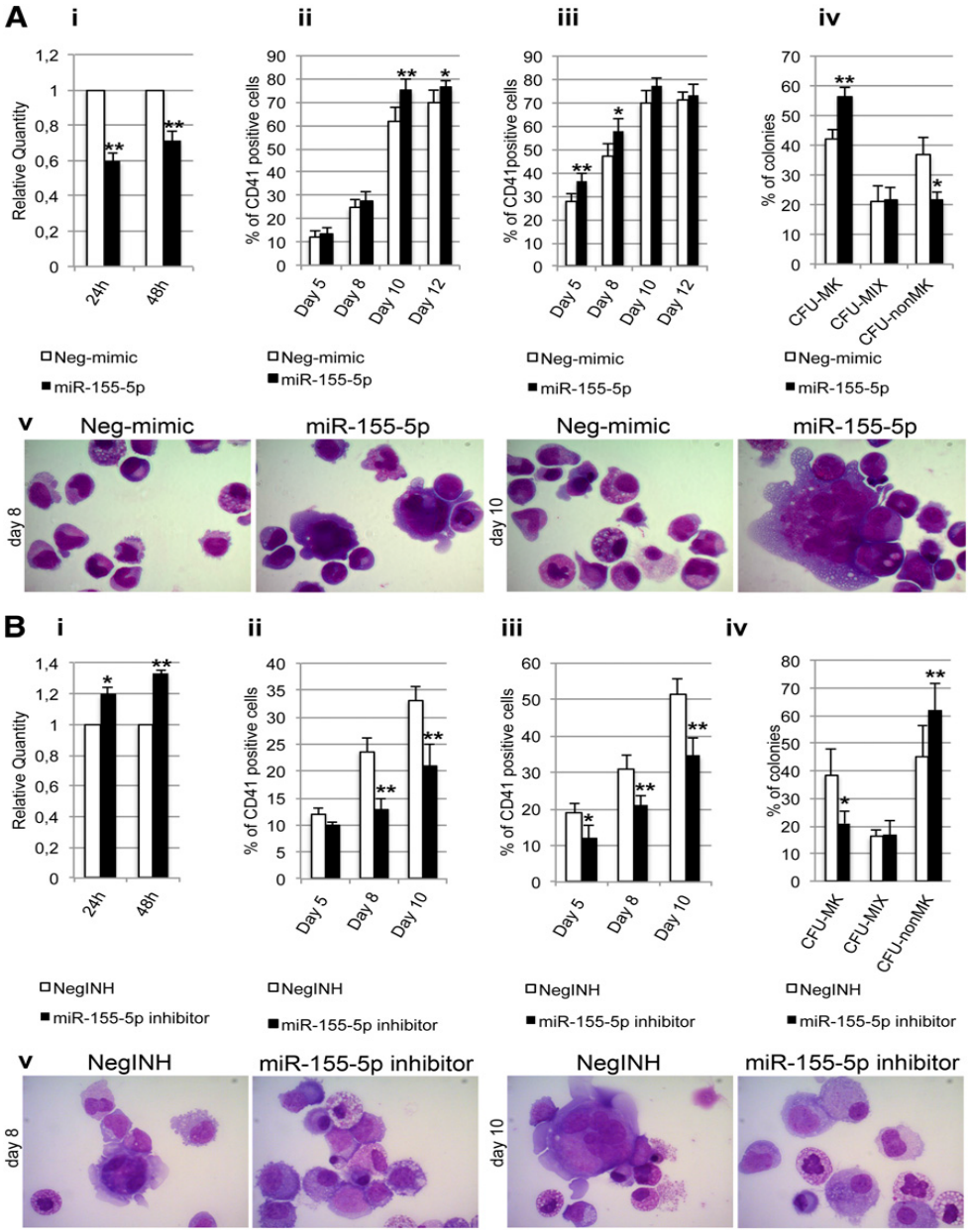


Fig. 21: Transfection of miR155-5p mimic and inhibitor in normal and PMF CD34^{+} cells. (A) Effect of miR155-5p mimic transfection on normal CD34^{+} cell differentiation. (i) Expression levels of JARID2 at 24 and 48 hours after the last nucleofection, measured by qRT-PCR and reported as RQ. (ii-iii) Results of statistical analysis on the percentage of CD41^{+} cells performed by flow cytometry at days 5, 8, 10, and 12 after the last nucleofection on serum-free multilineage and MK unilineage cultures. (iv) Results of the statistical analysis of the collagen-based clonogenic assay. The cells were plated 24 hours after the last nucleofection and scored after 12 days. (v) Morphologic analysis of Neg-mimic and miR-155-5p nucleofected cells after MGG staining at days 8 and 10 of MK unilineage serum-free liquid culture after the last nucleofection in a representative experiment. Values are reported as mean 6 SEM. **P<0.01 vs Negmimic;*P<0.05 vs Neg-mimic. The results come from 5 independent experiments. (B) Effect of miR155-5p downregulation in PMF CD34^{+} cells. (i)Expression levels of JARID2 in PMF CD34^{+} cells at 24 and 48 hours after the last nucleofection of miR-155-5p inhibitor. The JARID2 expression was measured by qRT-PCR, and data are reported as RQ. (ii-iii) Percentage of viable CD41^{+} cells assessed by flow cytometry at days 5, 8, and 10 after the last nucleofection on serum-free multilineage and MK unilineage cultures. (iv) Results of the statistical analysis of the collagen-based clonogenic assay. The cells were plated 24 hours after the last nucleofection and scored after 12 days. (v) Morphologic analysis of negative control inhibitor (NegINH) and miR-155-5p inhibitor treated cells after MGG staining at days 8 and 10 of MK unilineage serum-free liquid culture after the last nucleofection in a representative experiment. Magnification X1000. Values are reported as mean 6 SEM.**P< 0.01 vs NegINH; *P <0.05 vs NegINH. The results come from 4 independent experiments.

Furthermore, the collagen-based assay showed that miR-155-5p overexpression causes a significant increase in the CFU-MK percentage coupled with a strong decrease of non-MK colonies (Figure 21Aiv). Finally, the morphologic analysis of MGG-stained cytospins showed a remarkable enrichment in MK precursors at different stages of maturation in miR-155-5p–overexpressing cells compared with controls at days 10 and 12 after the last nucleofection (Figure 21Av). Furthermore, the study was aimed at assessing whether miR-155-5p downregulation in PMF $CD34^+$ cells could reduce the expansion MK lineage. First, it was evaluated JARID2 levels upon miR-155-5p silencing, observing a statistically significant increase at 24 and 48 hours after the last nucleofection (Figure 20Bi). Strikingly, knockdown of miR-155-5p impaired the ability of PMF $CD34^+$ cells to give rise to $CD41^+$ cells, in both multilineage and MK unilineage cultures (Figure 21Bii-iii). Those observations were further confirmed by decrease of CFU-MK colonies in collagen-based assays (Figure 21Biv) and by decrease of MK progenitors in MGG-stained cytospins (Figure 21Bv).

5.1.9 FUNCTIONAL VALIDATION OF THE MIR-155-5p/JARID2 AXIS IN MK LINEAGE EXPANSION

In order to demonstrate that miR-155-5p affects megakaryocytopoiesis by means of JARID2 modulation, it was concurrently overexpressed JARID2 and miR-155-5p in normal $CD34^+$ cells. To this aim, CB $CD34^+$ cells were nucleofected twice with miR-155-5p or Neg-mimic and consecutively transduced with the retroviral vector expressing JARID2 gene (LJARID2IΔN) or empty vector (LXIΔN) (see timing flowchart in Figure 22Ai). To assess JARID2 expression, qRT-PCR was performed after $NGFR^+$ cell purification (Figure 21Aii). As shown in Figure 21Aiii, the fraction of $CD41^+$ cells in the MK unilineage culture decreased in miR-155-5p/LJARID2IΔN compared with miR-155-5p/LXIΔN cells at days 4, 7, and 11 post purification. Moreover, the CFU-MK assay results showed that JARID2 overexpression in miR-155-5p cells causes a significant decrease in the CFU-MK percentage compared with miR-155-5p/LXIΔN cells (Figure 22Aiv). Furthermore, to confirm that miR-155-5p–driven megakaryopoiesis depends on JARID2 mRNA levels, it was simultaneusly silenced miR-155-5p and JARID2 in normal $CD34^+$ cells (see timing flowchart in Figure 22Bi). The expression level of JARID2 was assessed by qRT-PCR at 48 hours after the last nucleofection (Figure 22Bii). Flow cytometric analysis of $CD34^+$ expression performed on the MK unilineage culture at days 8, 10, and 12 after the last nucleofection showed a decrease in the MK fraction in miR-155-5p-silenced cells compared with NegCTR/negative control inhibitor (NegINH) cells (Figure 22Biii). As expected, the simultaneous JARID2 knockdown could rescue the MK differentiation unbalance in miR-155-5p silenced cells. In agreement with the flow cytometry data, the results of MK assay highlighted that the concurrent downregulation of miR-155-5p and JARID2 could prevent the impairment of MK differentiation observed in both independently silenced miR-155-5p and JARID2 samples (Figure 22Biv).

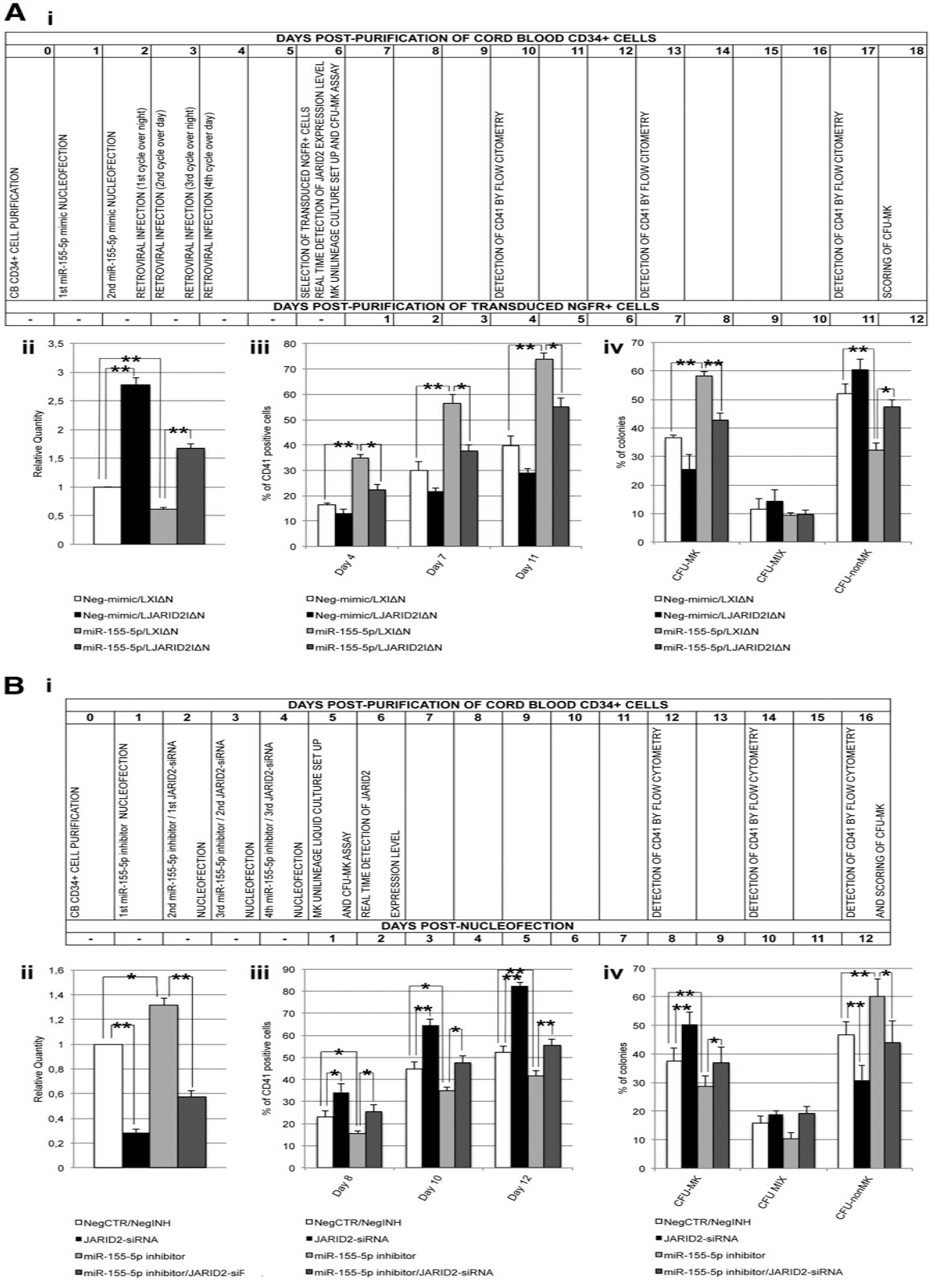


Fig. 22: Simultaneous overexpression or inhibition of JARID2 and miR-155-5p in normal CD34$^+$ cells. (A) Rescue of JARID2 expression in miR-155-5p–overexpressing cells. (i) Flowchart listing the experiment timing (expressed in days) after NGFR$^+$ cell purification. (ii) Expression levels of JARID2 in CB CD34$^+$ cells after NGFR$^+$ cells purification. The JARID2 expression was measured by qRT-PCR, and data are reported as RQ. (iii) Percentage of viable CD41$^+$ cells in the MK unilineage culture assessed by flow cytometry at days 4, 7, and 11 after NGFR$^+$ cell purification. (iv) Results of the statistical analysis of the collagen-based clonogenic assay. The cells were plated 24 hours after NGFR$^+$ cell purification and scored after 12 days. Values are reported as mean 6 SEM. **P<0.01; *P <0.05. The results come from 3 independent experiments. (B) Simultaneous downregulation of JARID2 and miR155-5p in CB CD34$^+$ cells. (i)

Flowchart reporting the experiment timing (expressed in days) after the last nucleofection. (ii) Expression levels of JARID2 in CB CD34$^+$cells at 48 hours after the last nucleofection. The JARID2 expression was measured by qRT-PCR and data are reported as RQ.
(iii) Percentage of viable CD41$^+$ cells in the MK unilineage culture assessed by flow cytometry at days 8, 10, and 12 after the last nucleofection. (iv) Results of the statistical analysis of the collagen-based clonogenic assay. The cells were plated 24 hours after the last nucleofection and scored after 12 days. Values are reported as mean 6 SEM. **P< 0.01; *P<0.05. The results come from 3 independent experiments.

All the results obtained in this first part of study, and reported in this thesis, were published in Plos One: *Norfo R., Zini R., Pennucci V., Bianchi E., Salati S., Guglielmelli P., Bogani C., Fanelli T.,* ***Mannarelli C.*** et al. *(2014) miRNA-mRNA integrative analysis in primary myelofibrosis CD34$^+$cells: role of miR-155/JARID2 axis in abnormal megakaryopoiesis. Blood. 2014 Sep 25;124(13):e21-32.*

5.2 CHARACTERIZATION THE NEW MIRNA AND MORNA EXPRESSION IN CD34$^+$ CELLS OF PMF PATIENTS.

Guglielmelli P, Bisognin A, Saccoman C, ***Mannarelli C****, et al. (2015).* PLoS ONE 10(10): e0140445.

5.2.1 SMALL RNA EXPRESSION IN CD34$^+$ CELLS OF PATIENTS WITH PMF.

It was considered small RNA sequencing data of 6 CD34$^+$ cells samples, including 3 samples collected from 3 pools of bone marrow CD34$^+$ cells of healthy subjects (CTR), and 3 samples of circulating CD34$^+$ cells of patients affected by primary myelofibrosis (PMF), two of which were from individual patients while the third constituted a pool obtained by mixing equal amounts of RNA from 4 PMF patients. The Illumina 2000 sequencing produced a total of 787, 913, 722 raw reads (131, 318, 954 per sample on average) that were deposited in Gene Expression Omnibus (GEO Series GSE69089).
It was detected a total of 917 sRNAs, including 784 know miRNAs, expressed in at least one of the 6 considered CD34$^+$ samples. Notably, 8 known miRNAs (miR-10a-5p, miR-181a-5p, miR-191-5p, miR-92a-3p, let-7a-5p, miR-146b-5p, miR-26a-5p and let-7f-5p) are highly expressed and contribute to the total miRNA expression throughout all samples from 2.5% to 25%, representing all together the 80% of the total expression. Moreover, it was detected 133 new sRNAs, including 34 new miRNAs produced from known hairpins, and 99 microRNA-offset RNAs (moRNAs). Table 25 reports a summary of different types of sRNAs detected in the considered samples, according to current miRNA annotations.

sRNAs	CTR	PMF	Total
known miRNAs	568	760	784
new miRNAs	20	31	34
moRNAs	52	96	99
Total new	72	127	133
Total	640	887	917

Tab. 25: Summary of small RNAs expressed in CD34$^+$ cells.

Statistical analyses was showed that patients and controls sRNAs profiles are significantly different from each other and highlight a characteristic miRNA and moRNA expression profile in PMF.

5.2.2 NEW MIRNAS IN CD34^{+} CELLS OF PMF.

In addition to 784 miRNAs annotated in miRBase, our in-house pipeline miR&moRe let us discover 34 new miRNAs expressed in considered samples **(see Table 26)**.

miRNA	Read Count		Strand	Position	Sequence
	CTR	PMF			
hsa-miR-107*	0	19	-	chr10:89592794-89592815	AGCTTCTTTACAGTGTTGCCTT
hsa-miR-1255a*	7	0	-	chr4:101330326-101330346	CTATCTTCTTTGCTCATCCTT
hsa-miR-1256*	13	29	-	chr1:20988343-20988363	CTAAAGAGAAGTCAATGCATG
hsa-miR-1276*	18	0	-	chr15:85770510-85770530	TGTCTCCACTGAGCACTTGGG
hsa-miR-1284*	6	4	-	chr3:71542003-71542021	GAAAGCCCATGTTTGTATT
hsa-miR-1289-1*	12	7	-	chr20:35454037-35454057	TGCAGACTCTTGGTTTCCACC
hsa-miR-1291*	0	10	-	chr12:48654462-48654490	ACTGTGGCTGTTGGTTTCAAGCAGAGGCC
hsa-miR-1294*	0	2	+	chr5:154347193-154347213	ACAGTGCCAACCTCACAGGAC
hsa-miR-1302-10*	0	1	-	chr15:101960531-101960550	TAGCATAAATATGTCCCAAG
hsa-miR-1302-11*	0	1	+	chr19:72019-72038	TAGCATAAATATGTCCCAAG
hsa-miR-1302-2*	0	1	+	chr1:30412-30431	TAGCATAAATATGTCCCAAG
hsa-miR-1302-9*	0	1	+	chr9:30190-30209	TAGCATAAATATGTCCCAAG
hsa-miR-1303*	0	3	+	chr5:154685789-154685810	AGCGAGACCTCAACTCTACAAT
hsa-miR-153-1-5p	10	19	-	chr2:219294165-219294185	GTCATTTTTGTGATCTGCAGC
hsa-miR-2110*	103	153	-	chr10:114174110-114174132	TCACCGCGGTCTTTTCCTCCCAC
hsa-miR-3155a*	16	7	+	chr10:6152207-6152228	CCTCCCACTGCAGAGCCTGGGG
hsa-miR-3648-1*	0	3	+	chr21:8208593-8208617	GTCGGCCGCGCTCGAGGGGTCCCCG
hsa-miR-3648-2*	0	3	+	chr21:8987119-8987143	GTCGGCCGCGCTCGAGGGGTCCCCG
hsa-miR-421*	0	42	-	chrX:74218427-74218448	CTCATTAAATGTTTGTTGAATG
hsa-miR-4424*	8	21	+	chr1:178677801-178677821	GTCCATTTCAAGTTAACTCTG
hsa-miR-4473*	0	21	-	chr9:20411202-20411225	CACTTGTAATGGAGAACACTAAGC
hsa-miR-451a*	0	4	-	chr17:28861384-28861404	TTTAGTAATGGTAATGGTTCT
hsa-miR-466*	22	0	-	chr3:31161754-31161774	TGTGTTGCATGTGTGTATATG
hsa-miR-548ag-2*	28	66	+	chr20:60564601-60564621	CAAGAACCTCAATTACCTTTG
hsa-miR-5696*	6	12	+	chr2:101309514-101309534	TCAGACTACCTAAATGAGCAC

hsa-miR-599*	0	6	-	chr8:99536688-99536707	TTTGATAAGCTGACATGGGA
hsa-miR-600*	5	2	-	chr9:123111598-123111618	CATAGGAAGGCTCTTGTCTGT
hsa-miR-641*	4	6	-	chr19:40282557-40282576	TGACTGTCCTATGTCTTTCC
hsa-miR-7854-5p	6	3	+	chr16:81533905-81533926	CTTCCATCTCCATCACCTTGAG
hsa-miR-941-1*	3	2	+	chr20:63919454-63919478	ACATGTGCCCAGGGCCCGGGACAGC
hsa-miR-941-2*	3	2	+	chr20:63919510-63919534	ACATGTGCCCAGGGCCCGGGACAGC
hsa-miR-941-3*	3	2	+	chr20:63919566-63919590	ACATGTGCCCAGGGCCCGGGACAGC
hsa-miR-941-4*	3	2	+	chr20:63919761-63919785	ACATGTGCCCAGGGCCCGGGACAGC
hsa-miR-941-5*	3	2	+	chr20:63919873-63919897	ACATGTGCCCAGGGCCCGGGACAGC

Tab. 26: New miRNAs discovered that are expressed in $CD34^{+}$. The Table reports expression (per sample group normalized read count), position and sequence of new miRNAs.

To find new miRNAs, it was considered all the hairpins precursors annotated in miRBase, to be used as reference for read mapping and small RNA detection and quantification. Some of the listed hairpin precursors were known to only generate one mature miRNA with only a handful of reads reported in miRBase across all NGS experiments surveyed. After identifying the hairpin region that would most likely pair with annotated mature it was classified as new miRNAs all the clusters of reads that map there. A consistent number of reads were attributed to new miRNAs. Since these reads passed stringent quality filtering and mapping criteria, it is improbable that they originate from sequencing errors. Furthermore, two of these new miRNAs, miR-2110* and miR-548ag-2*, resulted highly expressed and showed a mean expression over the median of expression values calculated on all small RNAs.

5.2.3 MORNAS DISCOVERY.

As anticipated, it was also detected in samples sequences aligning to hairpins outside known and novel miRNAs, that correspond to expressed microRNA-offset RNAs, called moRNAs. MoRNAs sequences partially overlap miRNA regions but generally span the Drosha cutting sites, letting us hypothesize a non canonical processing of the hairpin precursor in moRNA biogenesis (Bortoluzzi et al., 2012). A complete list of all the detected moRNAs is in Table 27.

moRNA	Read count		Strand	Position	Sequence
	CTR	PMF			
hsa-moR-101-1-5p	0	43	-	chr1:65058500-65058520	TGACTGACAGGCTGCCCTGGC
hsa-moR-103a-1-5p	0	13	-	chr5:168560965-168560982	AAGTTTTCTTACTGCCCT
hsa-moR-103a-2-3p	567	252	+	chr20:3917564-3917586	AAGAACCAAGAATGGGCTGCCCT
hsa-moR-103a-2-5p	117	37	+	chr20:3917484-3917502	AGCTGCGTCTTTGTGCTTT
hsa-moR-106b-3p	24	0	-	chr7:100093978-100094001	TCCAGCAGGGCACGCACAGCGTCC

hsa-moR-106b-5p	508	112	-	chr7:100094065-100094084	CCGCTCCAGCCCTGCCGGGG
hsa-moR-10a-5p	0	11	-	chr17:48579928-48579946	ATCTGTCTGTCTTCTGTAT
hsa-moR-1248-3p	0	12	+	chr3:186786787-186786804	AAAGACTGGGGTGGACCT
hsa-moR-125a-5p	11	65	+	chr19:51693247-51693266	ACCATGTTGCCAGTCTCTAG
hsa-moR-126-5p	27	8	+	chr9:136670596-136670614	CGCCTCCGCTGGCGACGGG
hsa-moR-128-1-5p	0	4	+	chr2:135665391-135665409	TGTTCCTGAGCTGTTGGAT
hsa-moR-128-2-3p	2493	0	+	chr3:35744548-35744568	CCCTACTGTGTCACACTCCTA
hsa-moR-136-5p	0	18	+	chr14:100884694-100884714	GTGTTGGATGAGCCCTCGGAG
hsa-moR-140-5p	18	10	+	chr16:69933083-69933101	TGTCTCTCTCTGTGTCCTG
hsa-moR-141-5p	0	14	+	chr12:6964093-6964111	CTGTCGGCCGGCCCTGGGT
hsa-moR-142-3p	32	13	-	chr17:58331218-58331244	TGAGTGTACTGTGGGCTTCGGAGATCA
hsa-moR-142-5p	0	27	-	chr17:58331305-58331332	CCGACGGACAGACAGACAGTGCAGTCAC
hsa-moR-146b-5p	0	3	+	chr10:102436498-102436518	AAGAACTTTGGCCACCTGGCA
hsa-moR-150-3p	59	4	-	chr19:49500772-49500797	GGGACCTGGGGACCCCGGCACCGGCA
hsa-moR-154-5p	0	2	+	chr14:101059749-101059767	TAGCGTGTGGTACTTGAAG
hsa-moR-155-5p	0	14	+	chr21:25573963-25573981	TTGCTGTAGGCTGTATGCT
hsa-moR-15a-5p	0	3	-	chr13:50049190-50049208	AATAAAACCTTGGAGTAAA
hsa-moR-15b-5p	46	57	+	chr3:160404586-160404605	TTTTGAGGCCTTAAAGTACT
hsa-moR-16-1-5p	263	424	-	chr13:50049050-50049069	ATAGCAATGTCAGCAGTGCC
hsa-moR-16-2-5p	47	31	+	chr3:160404733-160404752	ACTGACATACTTGTTCCACT
hsa-moR-17-5p	0	4	+	chr13:91350596-91350616	TTGTGACCAGTCAGAATAATG
hsa-moR-181a-2-5p	0	2	+	chr9:124692461-124692478	CCTTCAGAGGACTCCAAG
hsa-moR-18a-5p	9	32	+	chr13:91350736-91350754	ATGTTGAGTGCTTTTTGTT
hsa-moR-196b-5p	0	5	-	chr7:27169551-27169571	GCACCAGAACTGGTCGGTGAT
hsa-moR-19a-5p	0	16	+	chr13:91350884-91350902	TTTGTTTGCAGTCCTCTGT
hsa-moR-19b-1-5p	0	16	+	chr13:91351185-91351205	TACTGAACACTGTTCTATGGT
hsa-moR-20a-5p	0	44	+	chr13:91351049-91351070	TGATGTGACAGCTTCTGTAGCA
hsa-moR-214-5p	0	49	-	chr1:172138879-172138896	ACAGAGTTGTCATGTGTC
hsa-moR-21-5p	2946	2437	+	chr17:59841243-59841271	ACATCTCCATGGCTGTACCACCTTGTCGG
hsa-moR-222-5p	0	9	-	chrX:45747098-45747116	AAGGTGTAGGTACCCTCAA
hsa-moR-23a-3p	246	98	-	chr19:13836566-13836592	CCGACCCTGAGCTCTGCCACCGAGGAT

hsa-moR-24-1-5p	0	6	+	chr9:95086004-95086024	TGTCGATTGGACCCGCCCTCC
hsa-moR-24-2-5p	5971	1719	-	chr19:13836350-13836376	TGCCTGGCCTCCCTGGGCTCTGCCTCC
hsa-moR-25-5p	37	40	-	chr7:100093631-100093651	CCGGGACTGGCCAGTGTTGAG
hsa-moR-26a-2-5p	13	27	-	chr12:57824681-57824699	CCATAGAGGCTGTGGCTGG
hsa-moR-27a-5p	1752	312	-	chr19:13836510-13836534	CGAAGCCTGTGCCTGGCCTGAGGAG
hsa-moR-29c-5p	0	2	-	chr1:207801924-207801942	CCCATCTCTTACACAGGCT
hsa-moR-301a-5p	0	3	-	chr17:59151210-59151230	CTACTTATTACTGCTAACGAA
hsa-moR-30c-1-5p	0	7	+	chr1:40757279-40757298	CTATAACCATGCTGTAGTGT
hsa-moR-324-5p	67	47	-	chr17:7223366-7223384	CTGAGCTGACTATGCCTCC
hsa-moR-32-5p	24	19	-	chr9:109046295-109046313	TCTGCTTGCTCTGGTGGAG
hsa-moR-326-5p	165	561	-	chr11:75335169-75335193	CCGGAGCCTCATCTGTCTGTTGGGC
hsa-moR-331-5p	20	38	+	chr12:95308425-95308443	TGGTTTTGTTTGGGTTTGT
hsa-moR-338-5p	0	4	-	chr17:81125946-81125963	CCGCACGGCTGTCCTCTC
hsa-moR-361-5p	4	21	-	chrX:85903704-85903722	TTTTCCTGGGATTTGGGAG
hsa-moR-3651-5p	0	1266	-	chr9:92292537-92292565	ATGGACAGCTCTCCAGTGGATTCGATGGG
hsa-moR-370-5p	0	18	+	chr14:100911130-100911149	CGGGGCACAAGACAGAGAAG
hsa-moR-374a-5p	0	39	-	chrX:74287348-74287366	AAGAAATTTTACATCGGCC
hsa-moR-377-5p	0	49	+	chr14:101062031-101062055	CCGTGCTGATGTTTGACCCTTGAGC
hsa-moR-378a-5p	6	4	+	chr5:149732810-149732827	GTGACAGAGCCACCCAGG
hsa-moR-382-5p	0	33	+	chr14:101054296-101054314	TTTTCTGTGGTACTTGAAG
hsa-moR-421-3p	0	2	-	chrX:74218372-74218391	CTGCTCTGTGATCTCCATGG
hsa-moR-421-5p	848	2784	-	chrX:74218449-74218472	CCTAATCCGGTGCACATTGTAGGC
hsa-moR-4485-5p	0	17	-	chr11:10508315-10508332	AGTATTAGAGGCACCGCC
hsa-moR-4521-5p	24	5	+	chr17:8186930-8186948	CGAATCCCATCCTCGTCGG
hsa-moR-493-5p	0	3	+	chr14:100869048-100869073	TCGGGGCTCATTCTGGCCTCCAGGGC
hsa-moR-496-5p	0	5	+	chr14:101060575-101060594	CAAGTCAGGTACTCGAATGG
hsa-moR-503-5p	18	32	-	chrX:134546395-134546413	GCCCGCGCTCAGCCGTGCC
hsa-moR-505-5p	0	6	-	chrX:139924219-139924237	TAAATTGATGCACCCAGTG
hsa-moR-542-5p	9	237	-	chrX:134541424-134541442	ATGCACAGATCTCAGACAT
hsa-moR-545-5p	0	24	-	chrX:74287189-74287208	CCAGCCTGGCACATTAGTAG
hsa-moR-548b-5p	0	2	-	chr6:119069121-119069138	TATATATTTAGGTTGGCG

hsa-moR-551b-3p	11	41	+	chr3:168551935-168551962	AGGCTGTGAGAATAACTGCAATTTAGAG
hsa-moR-551b-5p	4	15	+	chr3:168551855-168551873	GATGTGCTCTCCTGGCCCA
hsa-moR-5695-3p	0	7	+	chr19:12920384-12920403	AATCTAGACAGATAGGCCTT
hsa-moR-6087-3p	0	2	+	chrX:109054577-109054601	CTTCTGGCGCCAAGCGCCCGGCCGC
hsa-moR-625-5p	0	2	+	chr14:65471097-65471114	TGGTAAGGGTAGAGGGAT
hsa-moR-671-5p	5	0	+	chr7:151238430-151238447	CTGGCAGGCCAGGAAGAG
hsa-moR-6724-1-5p	683	280	+	chr21:8205298-8205332	TGTGGGGGAGAGGCTGTCGCTGCGCTTCTGGGCCC
hsa-moR-6724-2-5p	683	280	+	chr21:8249488-8249522	TGTGGGGGAGAGGCTGTCGCTGCGCTTCTGGGCCC
hsa-moR-6724-3-5p	683	280	+	chr21:8388345-8388379	TGTGGGGGAGAGGCTGTCGCTGCGCTTCTGGGCCC
hsa-moR-6724-4-5p	683	280	+	chr21:8432513-8432547	TGTGGGGGAGAGGCTGTCGCTGCGCTTCTGGGCCC
hsa-moR-7-1-5p	213	171	-	chr9:83969836-83969859	CATTGGATGTTGGCCTAGTTCTGT
hsa-moR-766-5p	0	4	-	chrX:119646822-119646841	CAGGACCTGGGCTTGGGTGG
hsa-moR-769-5p	171	218	+	chr19:46018939-46018959	TGCTGATTCCTGGGCTCTGAC
hsa-moR-876-5p	0	9	-	chr9:28863698-28863716	ACACAAACTGTGAAGTGCT
hsa-moR-92a-1-5p	6	32	+	chr13:91351303-91351322	AACTCAAACCCCTTTCTACA
hsa-moR-93-5p	0	33	-	chr7:100093839-100093861	CTTGGACCTCAGTCCTGGGGGCT
hsa-moR-941-1-3p	3	2	+	chr20:63919510-63919534	ACATGTGCCCAGGGCCCGGGACAGC
hsa-moR-941-2-3p	3	2	+	chr20:63919566-63919590	ACATGTGCCCAGGGCCCGGGACAGC
hsa-moR-941-3-3p	3	2	+	chr20:63919622-63919646	ACATGTGCCCAGGGCCCGGGACAGC
hsa-moR-941-4-3p	3	2	+	chr20:63919817-63919841	ACATGTGCCCAGGGCCCGGGACAGC
hsa-moR-941-4-5p	6564	2531	+	chr20:63919746-63919768	CACCCGGCTGTGTGCACATGTGC
hsa-moR-941-5-3p	3	2	+	chr20:63919929-63919953	ACATGTGCCCAGGGCCCGGGACAGC
hsa-moR-941-5-5p	9780	3800	+	chr20:63919858-63919880	CACCCGGCTGTGTGCACATGTGC
hsa-moR-98-5p	0	9	-	chrX:53556322-53556340	GGATTCTGCTCATGCCAGG
hsa-moR-99b-5p	0	2	+	chr19:51692594-51692616	CCCGGACTCCTGGGTCCTGGCAC
hsa-moR-let-7a-1-5p	27	4	+	chr9:94175942-94175960	ATGTTCTCTTCACTGTGGG
hsa-moR-let-7a-2-5p	0	4	-	chr11:122146591-122146611	TTGTGACTGCATGCTCCCAGG
hsa-moR-let-7a-3-5p	0	2	+	chr22:46112732-46112750	AGACCGACTGCCCTTTGGG
hsa-moR-let-7b-5p	9	27	+	chr22:46113671-46113689	CAAGGCCGGGCCTGGCGGG

hsa-moR-let-7d-5p	15	18	+	chr9:94178821-94178839	AAAAAAATGGGTTCCTAGG
hsa-moR-let-7f-2-5p	22	46	-	chrX:53557269-53557287	GACACTGGTGCTCTGTGGG
hsa-moR-let-7i-5p	31	37	+	chr12:62603669-62603689	TCCCCGACACCATGGCCCTGG

Tab. 27: List of moRNAs expressed in considered CD34^{+} samples. The first seven moRNAs are expressed over the third quartile of all sRNAs expression.

Noteworthy, 28 moRNAs were highly expressed, 26 of them over the median of the short RNAs expression values distribution and 12 of them even over the third quartile **(Table 28)**.

moRNA	Average read count CTR	Average read count PMF	Strand	Position	Sequence
moR-128-2-3p	2493	0	+	chr3:35744548–35744568	CCCTACTGTGTCACACTCCTA
moR-21-5p	2946	2437	+	chr17:59841243–59841271	ACATCTCCATGGCTGTACCACCTTGTCGG
moR-24-2-5p	5971	1719	-	chr19:13836350–13836376	TGCCTGGCCTCCCTGGGCTCTGCCTCC
moR-27a-5p	1752	312	-	chr19:13836510–13836534	CGAAGCCTGTGCCTGGCCTGAGGAG
moR-3651-5p	0	1266	-	chr9:92292537–92292565	ATGGACAGCTCTCCAGTGGATTCGATGGG
moR-421-5p	848	2784	-	chrX:74218449–74218472	CCTAATCCGGTGCACATTGTAGGC
moR-6724-1-5p	683	280	+	chr21:8205298–8205332	TGTGGGGGAGAGGCTGTCGCTGCGCTTCTGGGCCC
moR-6724-2-5p	683	280	+	chr21:8249488–8249522	TGTGGGGGAGAGGCTGTCGCTGCGCTTCTGGGCCC
moR-6724-3-5p	683	280	+	chr21:8388345–8388379	TGTGGGGGAGAGGCTGTCGCTGCGCTTCTGGGCCC
moR-6724-4-5p	683	280	+	chr21:8432513–8432547	TGTGGGGGAGAGGCTGTCGCTGCGCTTCTGGGCCC
moR-941-4-5p	6564	2531	+	chr20:63919746–63919768	CACCCGGCTGTGTGCACATGTGC
moR-941-5-5p	9780	3800	+	chr20:63919858–63919880	CACCCGGCTGTGTGCACATGTGC

doi:10.1371/journal.pone.0140445.t002

Tab. 28. List of most abundant moRNAs in considered CD34^{+} samples, which are expressed over the third quartile of all sRNAs expression.

It was classified moRNAs on the basis of the hairpin precursor arm they where processed from: 5'-moRNAs mapping to the 5' hairpin arm, and 3'-moRNAs spanning over the 3' hairpin arm. 5' -moRs were significantly more abundant respect to 3' -moRs. Out of 99

moRNAs expressed in considered samples, only 16 (16.2%) were processed from the 3' hairpin arm, while 83 (83.8%) were 5' -moRs. According to our data, seven hairpins were processed producing two moRNAs each. 5'-moRs estimated expression values were 10 times higher than 3'-moRs, ranging from summed up normalized values over all samples of 5 to 40,739, compared to a 3'-moRs range of 6 to 7,478. Both 3'-moRs and 5'-moRs are more expressed in controls than in PMF patient samples. Considering the 9 most expressed moRNAs, Fig 23 A shows expression estimations in PMF and CTR samples of all the expressed small RNAs that are produced from the same hairpins precursor. Specific hairpins, as hsa-mir-421 and hsa-mir-941-1, reported in Fig 23 A , express two moRNAs each.

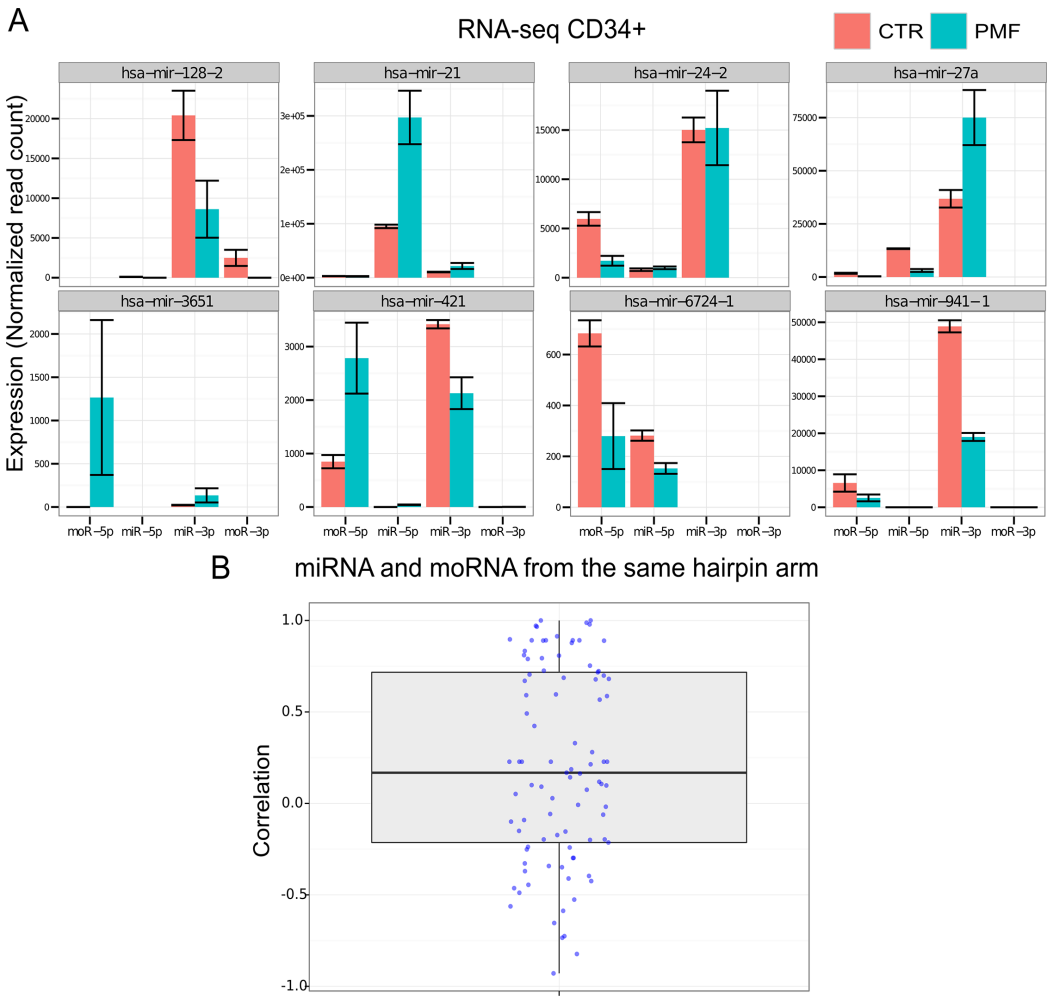


Fig.23 : A) Expression, in CD34^{+} cells of PMF patients and controls, of miRNAs and moRNAs produced from the same hairpin, considering the hairpins expressing most abundant moRNAs; the box plot in panel (B) shows the distribution of Pearson correlation values calculated pairwise between expression profiles of moRNAs and miRNAs produced from the same hairpin arm, considering all moRNAs detected in CD34^{+} cells.

Considering the miRNA and the moRNA produced from the same hairpin arm, it was noted that in 4 out the 9 reported in Fig 23 A the moRNA is more expressed than the miRNA and in one the miRNA close to the moRNA is not detected at all. Moreover, some hairpins show a similar trend toward up- or down-regulation for both the miRNA and the moRNA, whereas in others the two sRNAs show opposite behavior. It was then compared the expression behavior of all expressed moRNAs with that of the miRNA coming from the same hairpin arm. The boxplot in Fig 23 B shows the distribution of 93 Pearson correlation values calculated pairwise between expression profiles of detected moRNAs and miRNAs from the same hairpin arm: the values range from -0.93 to +1, with median and mean values of 0.17 and 0.21, indicating a very slight tendency toward positive correlation, the presence of abundant anticorrelated pairs (36,38.7%). Considering the statistical significance of correlation, only 7 pairs (7.5%) resulted significantly correlated (with a q-value at most 0.1); since they include an anticorrelated pair (5' moR-93 and miR-93-5p) and do not overlap with the group of highly expressed moRNAs, it is possible to conclude that moRNA expression is largely

independent from that of the close miRNA. Even if moRNAs mechanism of action is still unknown, their considerable high expression level in this dataset together with previously reported observations (Bortoluzzi et al., 2012; Gaffo et al., 2014; Langenberger et al., 2009) offers an indirect but intriguing indication of a biological role.

5.2.4 IDENTIFICATION OF sRNA DIFFERENTIALLY EXPRESSED IN PMF vs CTR.

It was recognized 37 sRNAs significantly differentially expressed (DE) in PMF patient samples respect to control $CD34^+$ (Table 29).

sRNA	Average normalized read count		log2 (FC)	p-value	Adjusted p-value
	CTR	PMF			
hsa-miR-1185-5p	0	136	15.00	1.24E-05	6.98E-04
hsa-miR-127-5p	0	317	15.00	4.66E-07	7.01E-05
hsa-miR-1277-5p	0	98	15.00	1.53E-04	6.28E-03
hsa-miR-299-3p	0	210	15.00	1.78E-04	6.69E-03
hsa-miR-323a-3p	0	121	15.00	5.34E-03	7.29E-02
hsa-miR-377-3p	0	206	15.00	9.34E-04	1.92E-02
hsa-miR-377-5p	0	155	15.00	8.32E-06	6.25E-04
hsa-miR-379-3p	0	89	15.00	2.76E-04	9.56E-03
hsa-miR-382-5p	0	205	15.00	2.97E-04	9.56E-03
hsa-miR-431-3p	0	80	15.00	1.72E-03	3.11E-02
hsa-miR-490-3p	0	719	15.00	2.48E-03	3.99E-02
hsa-miR-539-3p	0	204	15.00	1.37E-03	2.58E-02
hsa-miR-543	0	262	15.00	4.64E-04	1.40E-02
hsa-miR-654-5p	0	99	15.00	6.37E-04	1.60E-02
hsa-miR-656	0	78	15.00	8.90E-04	1.91E-02
hsa-miR-665	0	317	15.00	2.67E-03	4.15E-02
hsa-miR-758-3p	0	171	15.00	3.04E-03	4.56E-02
hsa-miR-873-5p	0	211	15.00	6.57E-04	1.60E-02
hsa-miR-25-3p	37	226192	12.59	2.85E-27	1.29E-24
hsa-miR-29a-3p	206	44817	7.77	6.73E-04	1.60E-02
hsa-miR-136-5p	34	1516	5.47	1.24E-04	5.57E-03
hsa-miR-495-3p	8	281	5.09	6.43E-04	1.60E-02
hsa-miR-873-3p	7	239	5.04	5.15E-03	7.27E-02
hsa-miR-485-5p	12	353	4.84	1.17E-03	2.30E-02
hsa-miR-19b-3p	3795	99173	4.71	7.13E-07	8.03E-05
hsa-miR-432-5p	14	343	4.62	2.09E-03	3.62E-02
hsa-5'-moR-542	9	232	4.62	6.72E-03	8.66E-02
hsa-miR-379-5p	31	561	4.16	7.86E-03	9.58E-02
hsa-miR-19a-5p	15	265	4.16	5.16E-03	7.27E-02
hsa-miR-33b-5p	26	394	3.90	6.09E-03	8.08E-02
hsa-miR-1307-5p	779	10914	3.81	8.23E-04	1.86E-02

hsa-miR-142-3p	5954	33867	2.51	7.73E-03	9.58E-02
hsa-miR-3150b-3p	196	7	-4.90	2.23E-03	3.73E-02
hsa-miR-10b-5p	11950	2855	-5.39	2.15E-08	4.84E-06
hsa-3'-moR-128-2	2489	0	-15.00	1.15E-05	6.98E-04
hsa-miR-128-2*	102	0	-15.00	1.87E-05	9.36E-04
hsa-miR-5008-3p	149	0	-15.00	4.17E-06	3.76E-04

Tab. 29: miRNAs and moRNAs significantly differentially expressed (DE) in PMF patient respect to control $CD34^+$, according to RNA-seq data.

Fig 24 A shows the logarithm of the mean expression ratio in PMF and control cells for DE miRNAs and moRNAs. While only five small RNAs are downregulated, the majority of DE sRNAs resulted upregulated in PMF patients. Noteworthy, among the differentially expressed sRNAs, 2 moRNAs (5' -moR-542 and 3' - moR-128-2) are included. Incidentally, the two miRNAs (miR-542-5p and miR-128-3p) that are produced by the same hairpin arm of differentially expressed moRNAs are not differentially expressed in the same sample comparison. 5' -moR-542 is up-regulated in PMF with a log2 FC of 3.5. 3'-moR-128-2 is highly expressed in normal $CD34^+$ cells, at levels over the third quartile of the overall small RNA expression distribution, and dramatically downregulated in PMF patients: the moRNA was not detected in considered PMF samples. It was mapped 3' -moR-128-2 sequence to the whole human genome to exclude multiple matching loci and to rule out mapping or annotations artifacts. Thus, It was possible to exclude that moRNA-associated reads could come from different or contaminating RNAs. An additional UCSC Blat (Kent et al., 2002) analysis confirmed that the moRNA sequence only aligned to a single genomic locus (chr3:35786042– 35786062). It is therefore confident that the detected small RNA is a moRNA derived from the non-canonical processing of the human mir-128-2 hairpin.

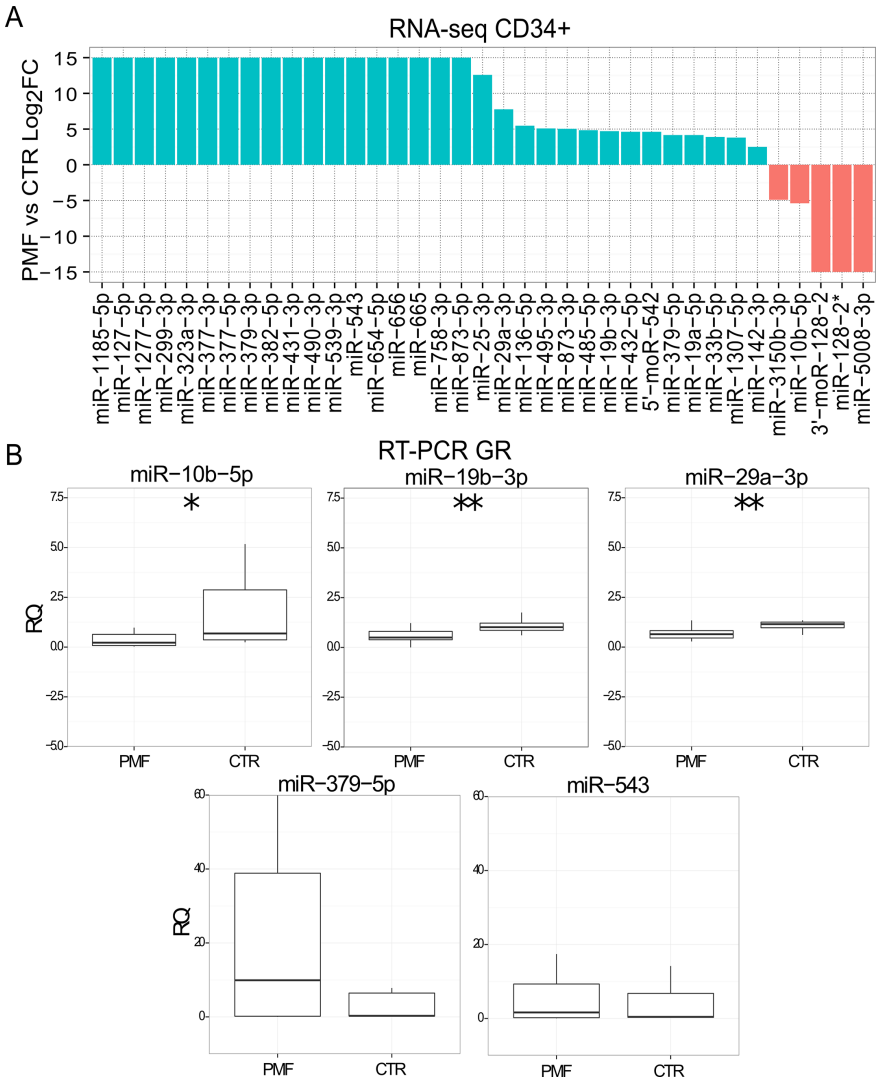


Fig 24: Differential expression of small RNAs in PMF vs CTR CD34^{+}. A) Log2 FC of 37 small RNA differentially expressed considering PMF vs CTR CD34+, according to RNA-seq data (FDR<0.05). When a small RNA was not expressed in one sample category, the ratio was infinite and we represent it as the arbitrary maximum value of 15. B) RT-PCR expression calculation of five selected miRNAs in granulocytes collected from an independent cohort of normal controls (n = 10) and of PMF (50) samples; ***, ** and * indicate respectively a P value <0.001, <0.01 or <0.05.

5.2.5 VALIDATIONS OF SELECTED DIFFERENTIALLY EXPRESSED MIRNAS IN PMF GRANULOCYTES.

It was selected the most significantly deregulated and highly expressed miRNAs for further analysis. Specifically, it was considered 6 miRNAs among the differentially expressed for a quantification with Real-time PCR (RT-PCR) in granulocytes collected from an independent and sizeable cohort of normal controls (N = 10) and PMF (N = 50), PV (N = 30) or ET (N = 30) patients. miR-10b-5p and moR-128-2 resulted significantly downregulated in PMF granulocytes samples, as according to RNA-seq data in CD34^{+} cells (Figs 24B and 25B).

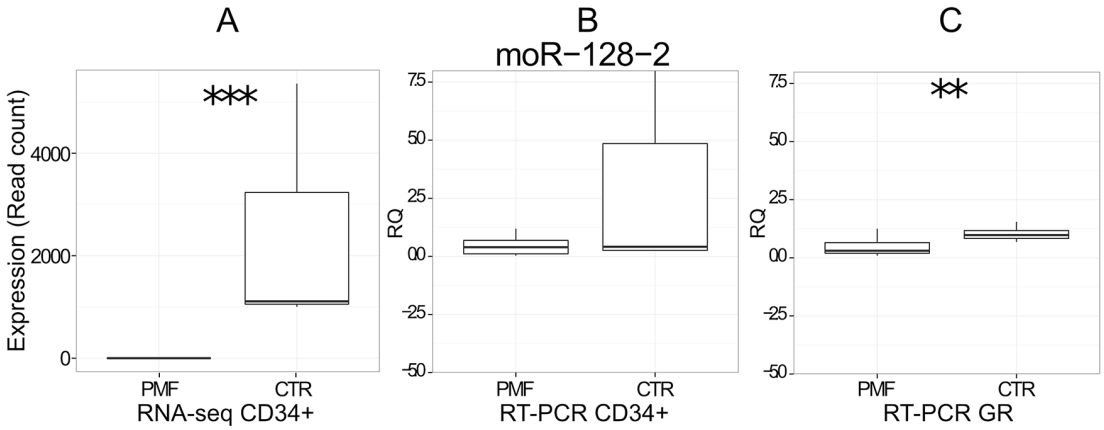


Fig. 25: Differential expression of 3'-moR-128-2 in PMF (n = 3) vs CTR (3) cells. A) moR expression in PMF and CTR $CD34^+$ according to RNA-seq data. B) RT-PCR expression (RQ) in $CD34^+$ cells from independent cohort of normal controls (n = 8) and of PMF (n=20) samples. C) RT-PCR expression (RQ) in granulocytes from independent cohort of normal controls (n = 10) and of PMF (50) samples; ***, ** and * indicate respectively a P value <0.001, <0.01 or <0.05.

Two additional miRNAs (miR-379-5p and miR-543) showed in PMF granulocytes the same trend toward upregulation in PMF granulocytes (Fig. 24B) as in $CD34^+$ cells by RNA-seq data. Both miR-29a-3p and miR-19b-3p resulted significantly downregulated in PMF granulocytes (Fig 24B). Furthermore, among the six small RNAs considered for validation in PMF granulocytes, 5 showed the same trend of changes also in ET or PV granulocytes, whereas miR-543 resulted upregulated in PMF and ET, and downregulated in PV, with small variations. miR-379-5p was upregulated in ET and in PV, as in PMF; miR-543 was slightly increased in ET, as in PMF, but not in PV; miR-19b-3p and moR-128-2 showed a similar trend, being decreased both in ET and in PV, but at a lower extent than in PMF; miR-10b-5p was decreased in ET and in PV, also more than in PMF. Notably, considering the statistical significance of the observed variation, miR-10b-5p is significantly downregulated both in PV and ET samples, indicating that it is associated with MPN in general more than being specific for PMF.

5.2.6 VALIDATION OF POTENTIAL mRNA TARGETS.

Bioinformatics predictions yielded several hundreds putative target genes for each selected small RNA (moR-128-2, miR-379-5p, miR10b-5p, miR-19b-3p, miR-29a-3p and miR-543).

Because only a few predicted targets have been experimentally validated in vitro or in vivo, and in order to narrow the analysis to a manageable number of variables, it was choose to focus on possible targets eventually selected based on their potential pathogenetic role in PMF. TaqMan® assays were carried out for 18 putative target genes in an independent cohort of $CD34^+$ cells from 20 PMF patients and 10 healthy subjects (Table 30). Using real-time RT-PCR, it was founded that TNSF10, MME, TCF4, TRPS1 and SYS1 were all significantly reduced in PMF $CD34^+$ compared with healthy controls, whereas BRCA1 and FKBP10 were increased. Other genes, probably due to the limited number of samples, did not reach a statistically significant difference yet they showed the expected trend of expression changes. In particular, it was founded that MECOM, MEIS1, AGO1, AGO3, RAN, CAV1, AKR1C1, TIMP3, FSTL1 were increased

in $CD34^+$ of PMF patients compared with normal subjects, while JAKMIP2 and PTPN4 were reduced.

According to miRNAs expression levels, and considering only significantly differentially expressed genes, an opposite behavior of expression variation was found for miR-379-5p and MME, TCF4 and SYS1; miR-19b-3p and miR-543 with TRPS1. Moreover, miR-10b-5p and FSTL1 showed opposite behavior and 5 tested genes (AGO1, RAN, MECOM, MEIS1, and CAV1) putative targets of moR-128-2 showed a trend toward increase in PMF, opposite to the moRNA. Table 30 reports results of mRNA expression tests, indicating, for each miRNA-mRNA pair, if the observed variations are in the same or in the opposite sense. However, before one can reliably conclude that these predicted interactions do play a role in PMF cell abnormalities and disease pathogenesis, functional studies of miRNA modulation will be required.

miRNA name	miRNA I/D	mRNA target gene name	mRNA I/D	mRNA DE p-value	Opposite variation
moR-128-2	D	MECOM	I	NS	YES
moR-128-2	D	MEIS1	I	NS	YES
moR-128-2	D	AGO1	I	NS	YES
moR-128-2	D	AGO3	I	NS	YES
moR-128-2	D	RAN	I	NS	YES
moR-128-2	D	CAV1	I	NS	YES
moR-128-2	D	TNSF10	D	0.0001	NO
miR-379-5p	I	MME	D	0.0001	YES
miR-379-5p	I	SYS1	D	0.0005	YES
miR-379-5p	I	TCF4	D	0.05	YES
miR-379-5p	I	BRCA1	I	0.05	NO
miR-379-5p	I	AKR1C1	I	NS	NO
miR-379-5p	I	TIMP3	I	NS	NO
miR-19b-3p	I	TRPS1	D	0.04	YES
miR-19b-3p	I	PTPN4	D	NS	YES
miR-19b-3p	I	FKBP10	I	0.02	NO
miR-29a-3p	I	AGO3	I	NS	NO
miR-543	I	TRPS1	D	0.04	YES
miR-10b-5p	D	FSTL1	I	NS	YES
miR-10b-5p	D	AGO3	I	NS	YES
miR-10b-5p	D	JAKMIP2	D	NS	NO
miR-10b-5p	D	PTPN4	D	NS	NO

Tab. 30. Expression pattern of 18 mRNAs potentially targeted by miRNAs, according to qRT-PCR data. For each mRNA, miRNA and moRNA, it was report if it is increased (I) or decreased (D) in the PMF vs CTR sample comparison; the mRNAs p-values associated to a significant difference are listed; the last column indicates if the expression of observed target mRNA variation is inversely related to the corresponding miRNA or moRNA.

All the results obtained in this second part of study, and reported in this thesis, were published in Plos One: *Guglielmelli P, Bisognin A, Saccoman C,* ***Mannarelli C****, Coppe A, Vannucchi AM, et al. (2015). Small RNA Sequencing Uncovers New miRNAs and moRNAs Differentially Expressed in Normal and Primary Myelofibrosis $CD34^+$ Cells. PLoS ONE 10(10): e0140445.*

5.3 EVALUATION OF PLASMA MIRNAS EXPRESSION PROFILE IN PMF PATIENTS.

5.3.1 MIRNAS EXPRESSION PROFILE IN PLASMA SAMPLES.

It was analyzed the expression of 175 miRNAs in RNA plasma samples collected from 25 patients with PMF and 6 healthy donors (Table 31). The miRNA detection experiments were performed by qRT-PCR on Serum/Plasma Focus microRNA PCR Panel.

Variable		Value
Number PMF	**Total**	25
Sex	**Male**	18
	Female	7
Age (yrs) at diagnosis: median (range)		65 (38-86)
Leukocyte count x 10^9/L : median (range)		8,08 (2,05-37)
Hemoglobin g/L: median (range)		10,40 (16-48,4)
Platelets x 10^9/L: median (range)		296,5 (65-1199)
Blasts (%): median (range)		1 (0-8)
Splenomegaly (n=23)	**Non palpable**	5
	palpable < 20 cm	12
	palpable > 20 cm	6
Bone marrow fibrosis grade at diagnosis (N=24)	**1**	9
	2	9
	3	6
IPSS (n=25)	**Total**	25
	low risk	3
	intermediate risk 1	8
	intermediate risk 2	5
	intermediate risk 3	9
Abnormal Karyotype		12

Median *JAK2V617F* Allele burden % (range)	50(0-100)
***JAK2* mutants**	12
***MPL* mutants**	1
***IDH1/2* mutants**	2
***DNMT3A* mutants**	1
***ASXL1* mutants**	7
***EZH2* mutants**	2
***SRSF2* mutants**	3
***CALR* mutants**	6
Constitutional sintoms	15
LAM	2
Deads	14

Tab.31: Laboratory and clinical parameters of 25 patients.

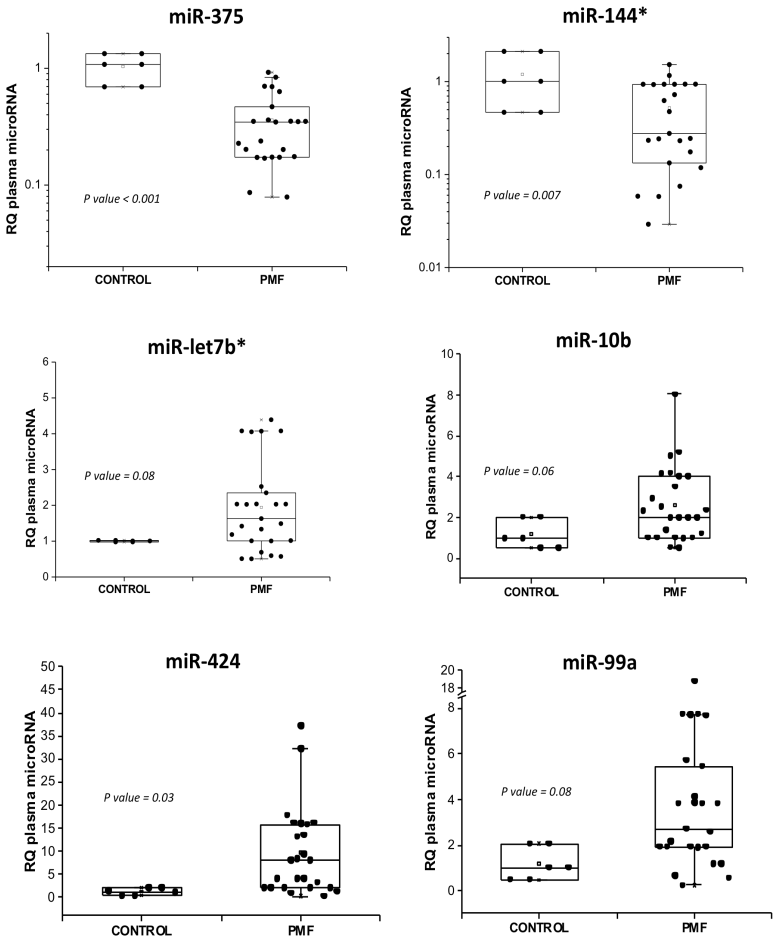


Fig. 26: miRnas resulted differentially expressed in PMF patients plasma compared to the controls.

It was identified differentially expressed miRNAs but only 6 of them were statistically different (P value<0.05): miR-let7b*, miR-10b-5p, miR-424 and miR-99a were up-regulated while miR-144* and miR-375 were down-regulated in PMF patients compared to the controls **(Fig. 26).** Of interest, miR-99a and miR-144* were reported to be deregulated in granulocytes of PMF patients (Guglielmelli et al.,2007).

5.3.2 CORRELATION BETWEEN MUTATIONAL STATUS, PHENOTYPIC CHARACTERISTICS AND MIRNA EXPRESSION.

From analysis of mutational status in this group of patients it was found 12 (48%) mutated In JAK2 gene, 1 (4%) in MPL, 2 (8%) in EZH2, 7 (28%) in ASXL1, 2 (8%) in IDH1/2, 3 (12%) in SRSF2 and 6 (24%) in CALR (Table 31). Comparing miRnas and mutational status of this genes we observed that there is a significative correlation only between the variation of miR-let-7b* expression and *ASXL1* mutational status, in fact patients with *ASXL1* mutation seem to have also greater expression of miR-let-7b* than *ASXL1* non-mutated (Fig. 27).

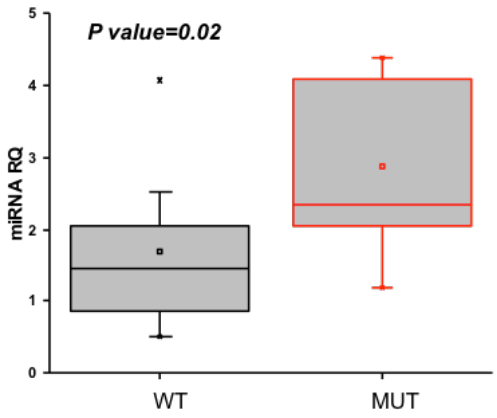


Fig. 27: *ASXL1* mutational status in relation to miR-let7b* expression.

Also, comparing miRNAs and clinical data it was found that BM fibrosis grade correlated with miR-let7b*, miR-10b-5p and miR-144*(P= 0.04, 0.02 and 0.02 respectively) (Fig.28): in particular, increased expression of miR-let7b* ad miR-10b-5p appears to be associated with a lower grade of fibrosis, on the contrary, the decreased expression of mir-144* appears to be associated with a BM fibrosis grade 3.

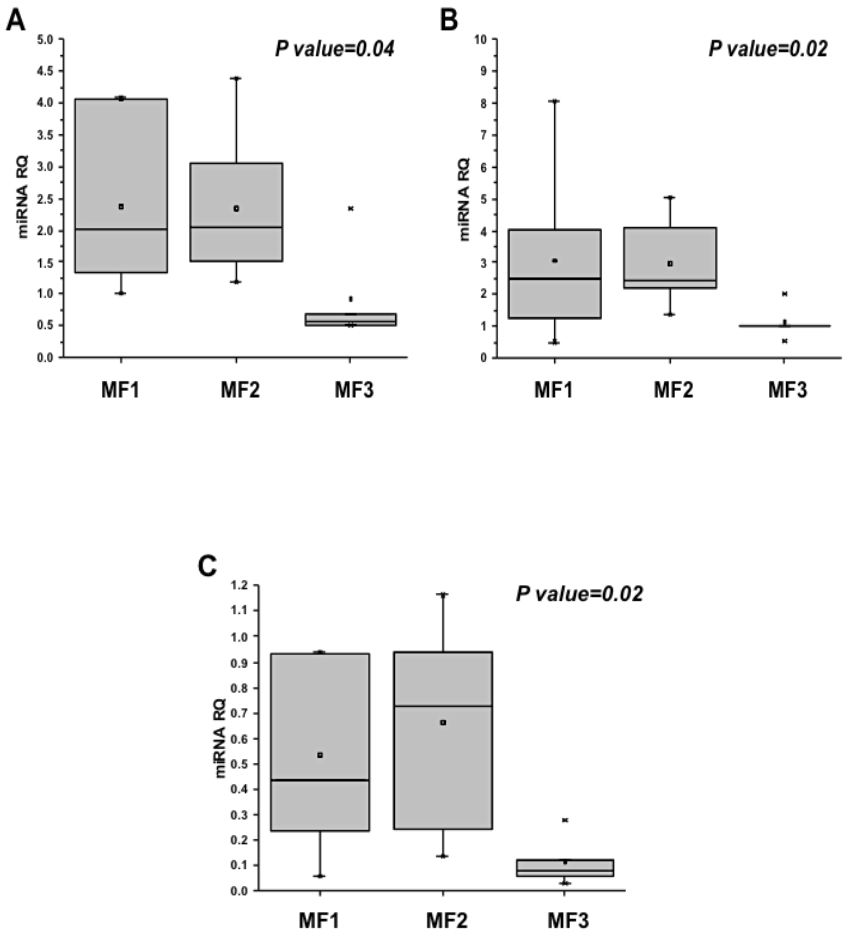
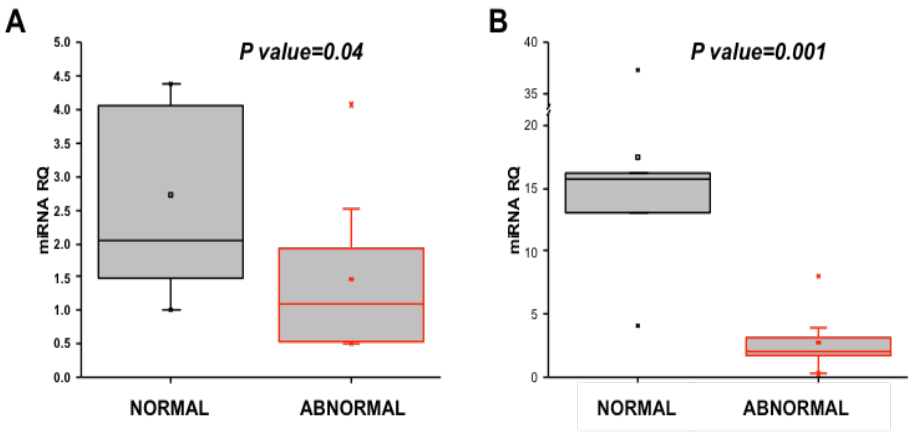


Fig.28: Correlation between BM Fibrosis grade with miR-let7b*(A), miR-10b (B) and miR-144*(C) expression.

Also, an abnormal karyotype was associated with miR-let7b*(p=0.04), miR-424 (p=0.001) and miR-99a (p=0.003): in particular, high levels of expression of these three miRna seem to be associated to a normal karyotype (Fig. 29).



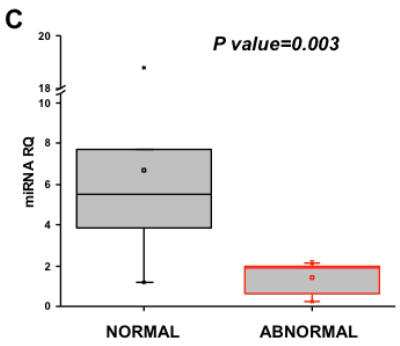


Fig.29: Correlation between abnormal Kariotipe and miR-let7b* (A), miR-424 (B) and miR-99a (C) expression.

Finally, the platelet count correlated directly only with miR-144* and miR-375 (p=0.03 and 0.02 respectively): a lower expression of these miRnas appears to be associated with lower platelet count (Fig.30).

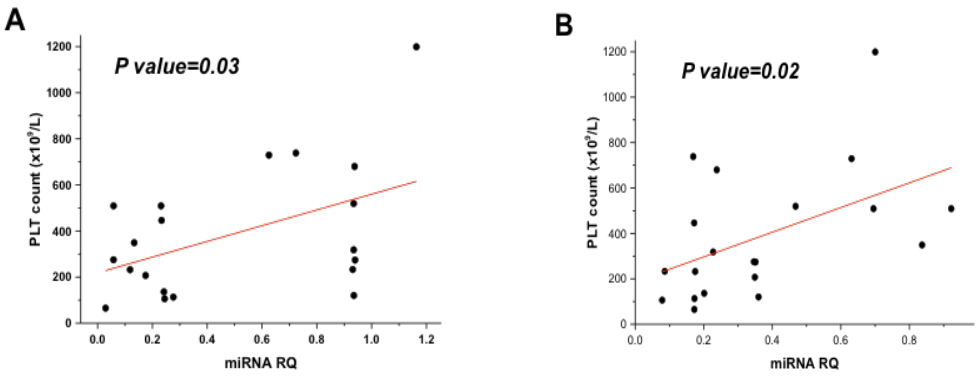


Fig. 30: Correlation between plateled count and expression of miR-144*(A) and miR-375 (B).

Progression to acute leukemia was observed in 2 (8%) patients and 14 (56%) patients was died but no impact of miRnas differentially expression on OS and leukemia transformation was seen.

5.4 MIRNAS EXPRESSION PROFILE IN *JAK2V617F KI* MOUSE MODEL.

5.4.1 STUDY OF MIRNAS PROFILE IN GR1$^+$ E TER119$^+$ CELLS.

In order to clarify the contribution of miRNAs to the pathogenesis of *JAK2V617F*-positive MPNs, it was analysed the miRNAs expression pattern in erythroid (TER119$^+$) and mieloid (GR1$^+$) cells purified from BM of *JAK2V617F* knock-in (L2) mouse model (2 KI and 2 WT) mice by using a combination of immunomagnetic and cell sorting approaches (Figure 31 A and B).

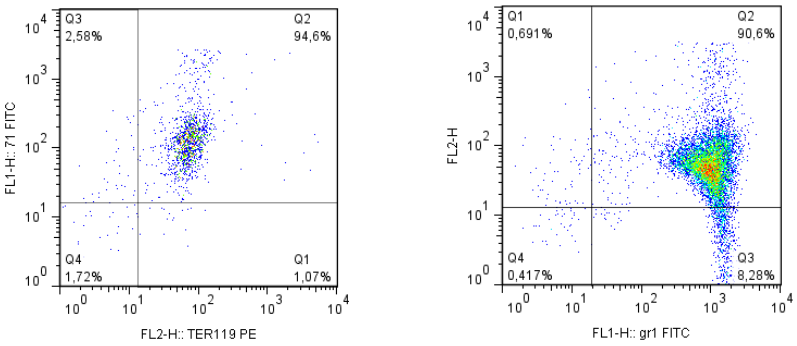


Fig. 31: Flow cytometry data plots of A) TER119$^+$and B) GR1$^+$.

Only samples with a purity greater than 90% were analyzed. In order to obtain sufficient quantity of miRNA for subsequent analysis it was necessary to extract total RNA from both L2 (TER119$^+$and GR1$^+$) cells and L- (TER119$^+$and GR1$^+$).Total RNA fraction was used to determine the RNA Integrity Number (RIN), which was in the range of 7.4 to 9.6, and to assess RNA concentration and rRNA ratio [28 s/18 s] by means of a bioanalyzer (Agilent®).

Once samples passed this quality control, the analysis of micro-RNA expression profile was performed by miRCURY LNA™ Universal RT microRNA PCR KIT (Exiqon®) that includes 384 miRNAs covering most well-characterized miRNAs in the miRNA Registry v14 (http://microrna.sanger.ac.uk). It was performed an initial data analysis using the software supplied with the real-time PCR instrument to obtain raw Ct values. Then the statistical analysis of results was carried out with the collaboration of the bioinformatic group of Biology Department of University of Padova, under the supervision of Dr S. Bortoluzzi (AGIMM). It was identified a list of differentially expressed miRNAs: 64 differentially modulated in TER119$^+$ (6 up-and 58 down-regulated) and 66 in GR1$^+$ (44 up-and and 22 down-regulated) (Fig.32).

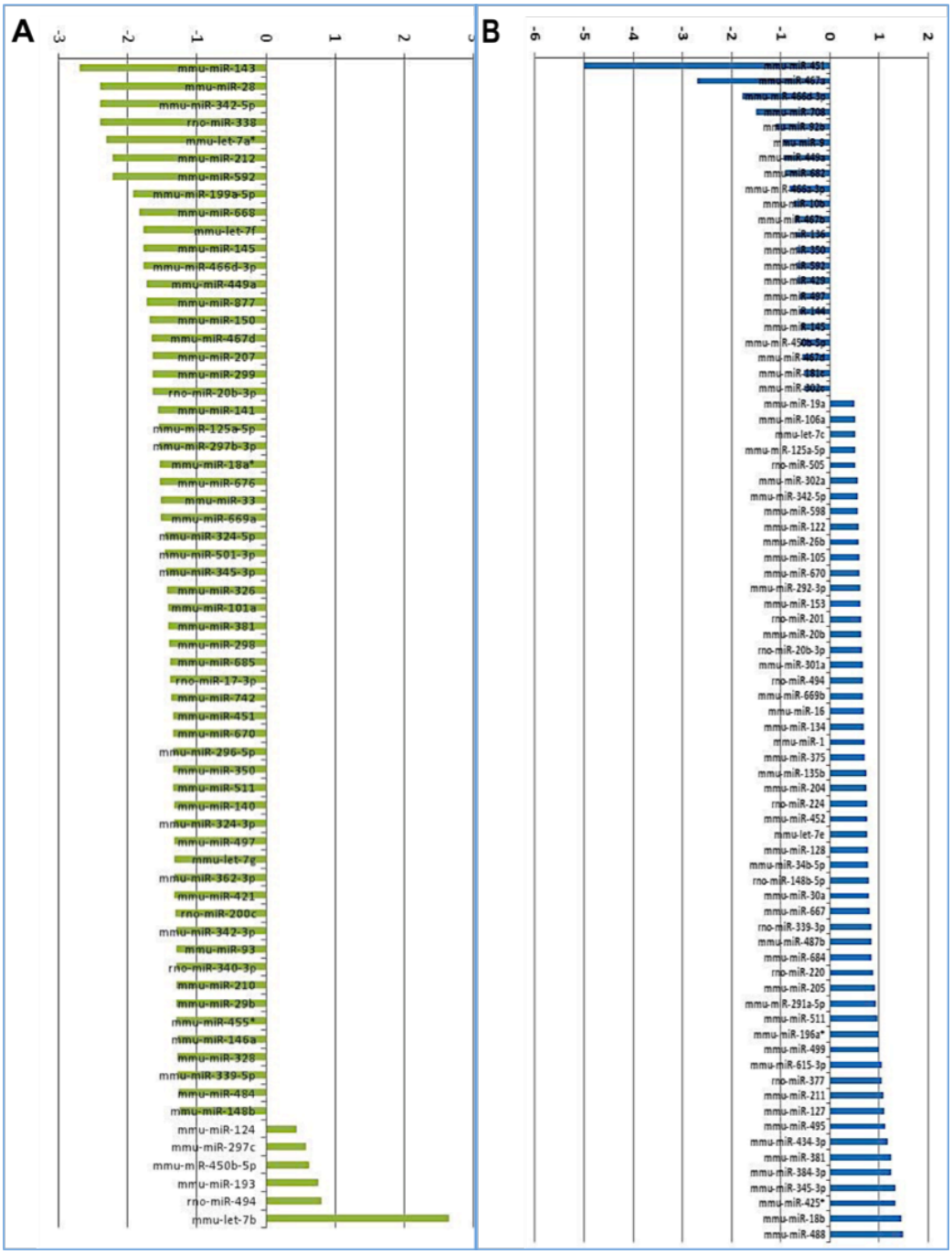


Fig.32: miRnas resulted differenzially expressed in First analysis In TER119+ (A) and In GR1+ (B) cells.

5.4.2 VALIDATION OF THE SELECTED MIRNAS.

Among them, the most differentially expressed were selected for validation: 5 were up-regulated (miR-147-3p, miR-15-5p, 193a-3p, miR-7a-5p, 291b-5p) and 7 down-regulated (miR-29b-3p, miR-467b-5p, miR-150-5p, miR-28a-5p, miR-592-5p, miR-484, miR-10a) (Table 32).

miRNA	CHROMOSOME	GR1	TER119
mmu-miR-147-3p	chr2: 122640803-122640881 [+]	UP	
mmu-miR-15b-5p	chr3: 69009772-69009835	UP	
mmu-miR-29b-3p	chr6: 31063023-31063093 [-]	DOWN	
mmu-miR-467b-5p	chr2: 10481248-10481320 [+]	DOWN	
mmu-miR-193a-3p	chr11: 79711969-79712034 [+]		UP
mmu-miR-7a-5p	chr13: 58392779-58392886 [-]		UP
mmu-miR-150-5p	chr7: 45121757-45121821 [+]		DOWN
mmu-miR-28a-5p	chr16: 24827855-24827940		DOWN
mmu-miR-10a-5p	chr11: 96317165-96317274 [+]		
mmu-miR-592-5p	chr6: 27936655-27936750 [-]		
mmu-miR-291b-5p	chr7: 3219482-3219560		
mmu-miR-484	chr16: 14159626-14159692 [+]		

Tab. 32: mRnas selected for the validation phase, relative chomosome location and expression. Pink boxes: up-regulated miRnas; Blu boxes: down-regulated.

Therefore, the expression level of selected miRNAs was evaluated in the same cells type of 5 JAK2V617F KI and 5 JAK2 WT mice by TaqMan® miRna assays.
It was confirmed up-regulation of miR-7a-5p and miR-291b-5p and down-regulation of miR-150-5p in $GR1^{+}$; in $TER119^{+}$, up-regulation of miR-7a-5p and down-regulation of miR-150-5p and miR-592-5p (Table 33).

miRna	GR1+ $JAK2^{V617F}$ KI vs WT	TER119+ $JAK2^{V617F}$ KI vs WT
miR-7a-5p	up	up
miR-291b-5p	up	nd
miR 150-5p	down	down
miR 592-5p	nd	down

Tab 33: MiRnas confirmed up and/or down regulated in the validation phase. nd: no different expression.

Moreover, in not-validated miRNAs it was found significant up-regulation of miR-147-3p, miR-467-5p in TER119^{+} and of miR-193-3p, miR-28a-5p, miR-484 and miR-10a-5p in GR1^{+}. Only 2 differentially expressed miRNAs were shared by both erythroid and myeloid cells (miR-7a-5p and miR-150-5p, up and down-regulated respectively), suggesting a direct relationships between these miRNAs and *JAK2V617F*.

5.4.3 MIRNAS TARGET PREDICTION ANALYSIS.

Potential mRNA targets were predicted in silico according to Targetscan 6.1. The TargetScan Human resource provides miRNA target predictions based on sequence complementary to target sites with emphasis on perfect base-pairing in the seed region and sequence conservation. These web-based computational tools are based on different algorithms which are based on several parameters calculated individually for each miRNA. These target prediction analysis included genes involved in pathways having functional relevance for MPN such as mTOR signaling pathway (PIK3R1 and EIF4B), CXCR4 pathway (CXCR4 and PTK2), cell cycle (e.g. TP53, MDM2, RB1, E2F1 and CDK2), apoptosis (i.e. CASP9, BCL2 and BCL2L1), epigenetic regulation (i.e. EZH1 and DNMT3A) and transcriptional modulation (e.g. SMAD1, NOTCH1 and MYB).

6 DISCUSSION

The molecular basis of MPN has been appreciated recently with the description of mutations in *JAK2, MPL, CALR* and other genes but several aspects of their pathogenesis remain elusive. In particular, the mechanisms and/or the molecules involved in the phenotypic mimicry of these disorders, on the basis of an unique mutational event, are largely unknown. In addition to this, because miRNAs were recently demonstrated to be deregulated in MPN cells (Guglielmelli et al., 2007; Lin et al., 2013), interest regarding their putative involvement in pathogenetic mechanisms is progressively growing. However, most of the miRNA and mRNA expression studies performed to date have been conducted using terminally differentiated hematopoietic cells, namely granulocytes or MKs (Guglielmelli et al., 2007; Slezak et al., 2009; Hussein et al., 2009). Because MPNs are considered to arise from the HSC compartment, understanding of the pathogenetic molecular mechanisms might be best assessed by studying $CD34^+$ cells.

So far, only 2 studies have reported data on miRNA expression profiling in a very small number of MPN $CD34^+$ samples (Lin et al., 2013; Zhan et al., 2013) and no integrated miRNA-mRNA expression analysis was available until now. For these reasons, in the fist part of this work, I performed an extensive study that concurrently profiled both gene and miRNA expression in the same $CD34^+$ cells sample from PMF patients by MicroArray technique. GEP and miEP analysis showed that PMF $CD34^+$ cells present a different expression pattern compared with BM and unmobilized PB $CD34^+$ cells. Differential expression analysis enabled the identification of several deregulated miRNAs and mRNAs suitable as biomarkers or as putative molecular targets for diagnostic or prognostic purposes. Thereafter, the most upregulated genes and miRNAs were monitored in PMF granulocytes because these cell could represent a more suitable source for clinical practice, whereas secreted proteins were assessed in the patients' sera. In this work, It was identified a set of 5 genes (ie, *LCN2, OLFM4, ANXA3, FGR,* and *LEPR*) and 6 miRNAs (ie, miR-19a-3p, miR-335-5p, miR-376c-3p, miR-379-5p, miR-487b-3p, andmiR-494-3p) whose expression levels are aberrant in PMF granulocytes as well as in $CD34^+$ cells. Evidence in PMF of a higher mRNA expression of the leptin receptor (LEPR), previously reported in AML (Konopleva et al., 1999), as well as of Src kinase FGR (Dos Santos et al., 2013) could also be useful hints to drive the future design of targeted drugs. Of note, LEPR and Src kinase inhibitors are already being used in preclinical or clinical trials (Shiplman et al., 2011; Tokuhisa et al., 2014). In addition it was demonstrated that OLFM4 and LCN2 secreted protein levels could be considered as PMF biomarkers because they were significantly higher in patients' sera compared with those of healthy controls. Strikingly, >80% of the PMF patients presented with higher OLFM4 protein levels compared with all the evaluated controls. The first part of this work has mainly provided information about the molecular mechanisms underlying PMF pathogenesis. Indeed, data analysis clearly showed that several genes involved in adhesion or migration processes (TM4SF1, RHOB, ARHGAP18, and MMP8) as well as fibrogenic potential (LEPR, MMP9, and TIMP3) are deregulated

in PMF CD34$^+$ cells. Interestingly, regulators of megakaryocytic commitment were also upregulated (ie, NFE-2, MEF2C, miR-146b-5p, and miR-34a-5p). Furthermore, it was found an increased expression of genes or miRNAs with oncogenic potential, such as CEACAM8, ANGPT1, miR-29a-3p, and miRNAs belonging to the miR-17-92 cluster (Schotte et al., 2012; Lemoli et al., 2009). Because one of the main mechanisms through which miRNAs act is degradation of specific targets (Ameres et al., 2013) miRNAs and their targets are expected to display anticorrelated expression. Hence, an Integrative Analysis (IA) approach was used to select the most reliable interactions.

As chromatin remodeling is one of the main processes involved in PMF pathogenesis, it was determined whether a DEM could affect this pathway. It was found that chromatin remodeler genes such as PHC3 and HMGB3 could be downregulated by a network of upregulated targeting miRNAs (ie, miR-18a-5p, miR-433-3p, miR-195-5p, miR-200c-3p, and miR-152-3p). It was also observed that low expression of different hematopoietic transcription factors as well as leukemia suppressors (ie, CEBP, FOXO1, and MLL5) is, at least in part, caused by several highly expressed oncomiRs such as miR-21-5p and miR-29a-3p. Finally, it was found a single regulatory network collecting the highest number of oncomiRs and target genes involved in malignant. Of note, by means of 3'UTR luciferase reporter assays, 11/17 (64.7%) putative interactions were confirmed for the previously mentioned network, highlighting the good predictive power of IA in identifying true miRNA-target interactions. Specifically, the upregulation of several targeting miRNAs explains the negative regulation of genes like CDC42 and NR4A3, whose downregulation leads to myeloproliferative disorders in murine models (Ramirez-Herrick et al., 2011; Yang et al., 2007) as well as HMGB3, which codes for a regulator of the self- renewal/differentiation balance in murine HSCs (Nemeth et al., 2006). Moreover, **up-regulated miR-155-5p, miR-195-5p, and miR-152-3p share the experimentally observed target *JARID2***, a chromatin remodeler that is a member of the Jumonji family of transcription factors belonging to the polycomb repressive complex 2 (Peng et al., 2009). Of note, Puda and colleagues demonstrated that JARID2 is frequently deleted in leukemic transformation of chronic myeloid malignancies (Puda et al., 2012). However, although the function of JARID2 in hematopoiesis has been already partially unraveled in mouse embryo development studies, there are no data about its role in human primary hematopoietic CD34$^+$cells (Kitajima et al., 1999). Thus, it was decided to investigate the effect of JARID2 downregulation in hematopoiesis by means of RNAi-mediated silencing in human normal CD34$^+$ cells. The findings of this work support the contributing role of JARID2 deficiency in the expansion of the MK lineage both in liquid and in semisolid culture. Because the most effective miRNA in downregulating *JARID2* 3'UTR-luciferase activity was miR-155-5p, whose enforced expression causes a myeloproliferative disorder in mice (O'Connell et al., 2008) this miRNA was accordingly overexpressed in hematopoietic CD34$^+$cells. These experiments lead to show that **miR-155-5p overexpression is implicated in the downregulation of *JARID2* mRNA**, thereby promoting the expansion of the MK compartment. In parallel, miRNA inhibition in PMF CD34$^+$ cells clearly confirm a link between **miR-155-5p and the increased megakaryopoiesis observed in PMF patients**.

Finally, the restoration of *JARID2* expression level in miR-155-5p–overexpressing $CD34^+$ cells impaired the expansion of MK lineage induced by miR-155-5p; this unbalance could be prevented by simultaneusly silencing *JARID2* and miR-155-5p as well, thus definitively demonstrating that miR155-5p affects megakaryopoiesis via *JARID2* modulation. Overall, this interaction could explain the MK hyperplasia observed in BM biopsies of PMF patients and the high proliferative potential of MKs derived from PMF $CD34^+$ cells reported in studies in vitro (Balduini et al., 2011). Moreover, the analysis of miR-155-5p and JARID2 mRNA levels in ET $CD34^+$ cells revealed that this regulatory axis specifically works in PMF $CD34^+$ cells. Taken together, integration of GEP and miEP uncovered regulatory networks in which aberrantly expressed miRNAs and genes interact, thereby elucidating some of the pathogenetic characteristics of PMF. Finally, IA has proved to be a good approach for identifying reliable mRNA/miRNA interactions that could contribute to PMF pathogenesis, such as the JARID2-miR-155-5p axis, which is involved in hyperplastic megakaryopoiesis.

In order to corroborate data obtained in the first part of this work regarding deregulation of miRNAS as well as with the aim to discover new miRnas, isomiRna and moRnas in PMF cell, a RNA-seq technique was employed to comprehensively profile small RNA expressed in PMF $CD34^+$ cells compared with control $CD34^+$ cells .

Such an approach enable the detection of **784 know miRNAs** expressed in $CD34^+$ cells and the discovery of **34 new miRNAs** produced from known hairpins. It was showed that expressed miRNAs with unique sequence are rare and that most miRNAs are isomiR mixtures, whose expression needs to be considered when dealing with overall miRNA expression estimation and for differential expression detection. Moreover, a set of **99 microRNAoffset RNAs (moRNAs)** probably produced from alternative microRNA precursors was discovered. Cluster analyses and heatmaps of samples demonstrated that patients'and controls' small RNAs profiles are significantly different and highlighted a characteristic miRNA and moRNA expression profile in PMF. In fact, according to RNA-seq data, it was identified that several sRNAs are expressed at significantly different level in patients' compared to controls' $CD34^+$. Subsequent analysis in granulocytes from independent patients and controls samples strengthened the evidence of 6 differentially expressed sRNAs, including **3'-moR-128-2.**

In the group of the miRnas with statistically significant different expression, of interest, is **miR-10b-5p** that resulted downregulated both in PMF $CD34^+$ and granulocytes. Accordingly, **miR-10b-5p** has been previously reported to be deregulated in breast cancer (Gee et al., 2008; Chan et al., 2013) and involved in chemoresistance related pathway (Ouyang et al., 2014). It has been validated as downregulated in endometrial carcinoma (Tsukamoto et al, 2014) bladder cancer (Zaravinos et al., 2012), advanced stage of small cell carcinoma of the cervix (SCCC) (Huang et al., 2012) and i clear cell renal cell carcinoma (ccRCC) and its expression level has been also included in a linear model that captures the metastatic tumor signature and patient prognosis (Wu et al., 2012).

miR-29a-3p was found upregulated in $CD34^+$ from PMF patients, and further validated by RT-PCR in an independent set of $CD34^+$ cells from 10 PMF patients and 8 healthy

subjects, by Rna-seq analysis confirmibg also data with RNA array. Of interest, **miR-29a-3p** resulted **upregulated in patients CD34$^+$** in respect to controls, **but downregulated in the same comparison when considering mature granulocytes.** This observation is also in accordance with results previously obtained by Han et al. (Han et al., 2010) who showed that miR-29a-3p is expressed at lower levels in hematopoietic progenitors compared to lineage-committed progenitors, including granulocytes, overall indicating that its expression level increases along with commitment. Therefore, the seobservations point to miR-29a as a deregulated small RNA in PMF, whose expression is uniquely modulated along myeloid differentiation of PMF CD34$^+$ cells. Consistently with our finding, Han et al. also showed also that sustained expression of miR-29a-3p in mouse HSC/progenitors pushed myeloid progenitors to self-renewal capacity, to biased myelopoiesis and to the onset of a myeloproliferative disorder that progressed to acute myeloid leukemia. Althougt in the first part of this work also the upregulation of miR-379-5p, miR-543 and miR-19b-3p was validated in an independent set ofPMF CD34$^+$, the RNA-seq data were conversely unable to confirm a statistically significant upregulation of these miRNAs in PMF, although there was a trend for miR 379-5p and miR-543. Interestingly, previously, validated microarray-based observations using two sets of qRT-PCR experiments, conducted on CD34$^+$ cells and on granulocytes, showing that PMF-specific variations of a few miRNAs may be observed in CD34$^+$ cells unlike in granulocytes. Indeed, miR-486-3p was significantly downregulated in PMF granulocytes and upregulated in PMF in CD34$^+$ cells. **In summary, available evidence indicate that miR-10b-5p and moR-128-2 are downregulated in PMF CD34$^+$ cells, whereas miR-19b-3p, miR-379-5p and miR-543 are upregulated.** For these miRNAs the evidence of differential expression in PMF was robust, since it was detected by NGS and also validated technically (by qRT-PCR) and biologically (in independent samples).

Regarding the newly discovered **3'-moR-128-2**, it was investigated in detail the sequence, structure, expression and possible functions, also by comparison with the cognate miR-128-3p; results showed that the miRNA and moRNA expression profiles are poorly correlated. At the functional level, assuming that moRNAs may act as miRNAs, it was noticed that 3'-moR-128-2 and miR-128-3p potential target genes and pathways are markedly different.

Interestingly, 3'-moR-128-2, that was expressed in normal CD34$^+$ cells and resulted absent in PMF cells, may target pathways related to the control of cell growth and proliferation and, strikingly, target several genes involved in microRNA biogenesis or in miRNA-mediated silencing. Its expression level was measured with qRT-PCR both in CD34$^+$ cells and in granulocytes. RT-PCR validation in CD34$^+$ cells were conducted considering 8 normal controls and 20 PMF patients, whereas the validation in granulocytes was done with the same design and samples used for miRNA validation**. A consistent downregulation of 3'-moR-128-2 was found in PMF patients according to RNA-seq and RT-PCR data in CD34$^+$ and to RT-PCR data in granulocytes.** Moreover, 3'-moR-128-2 was decreased, but at a lower extent, without reaching statistical significance, also in PV and ET granulocytes. Intrigued by the striking expression

pattern of the newly discovered 3'-moR-128-2 in collaboration with University of Padua group it was looked in details into sequence, structure, expression and functional differences of 3'-moR- 128-2 and miR-128-3p.

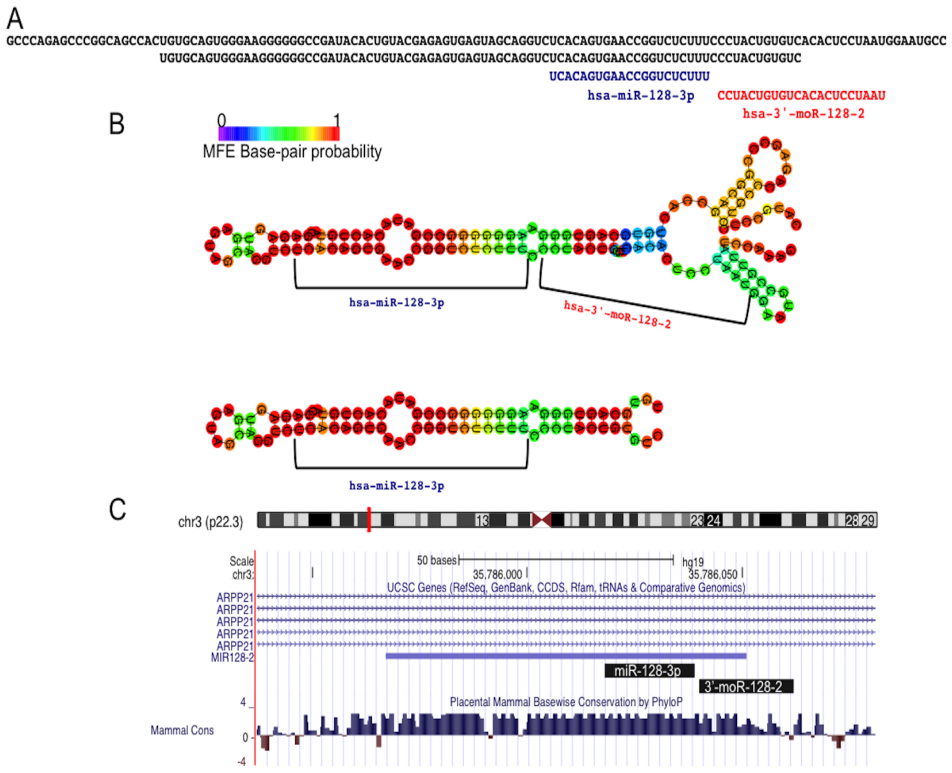


Fig 33: The 3'-moR-128-2 is produced by the precursor sequence of miR-128-3p. A) The moRNA is derived from a region of the primary miRNA sequence exceeding the canonical hairpin precursor sequence, and it is not exaclty adjactent to the annotated miRNA. B) Minimum free energy (MFE) folding structure, predicted by RNA fold, for the canonical hairpin sequence and for the longer one, from which the moRNA is probably derived.C) Both the considered small RNAs are conserved in evolution through vertebrates.

In **Fig 33** we show additional sequence information regarding miR-128-3p and 3'-moR-128-2. Since the moRNA sequence is not contained in the canonical hairpin (Fig 33A), the moRNA probably derives from the processing of an alternative hairpin precursor. In Fig 33B it was showed the RNA fold predicted minimum free energy (MFE) folding structure of the canonical hairpin and of a longer sequence from which the moRNA can be derived. Fig 33C shows that mir-128 locus is inside an intron of ARPP21 gene, and displays the genomic region Mammals UCSC base-wise PhyloP conservation score. moRNA biological roles and mechanisms of function still deserve investigation. Very likely, moRNAs can function as miRNAs in post-transcriptional gene silencing, guiding RISC to complementary target mRNAs. This was first demonstrated by Umbach and colleagues, that used a luciferase-based indicator assay to demonstrate that a viral moRNA (moR-rR1-3-5p) has inhibitory activity against an artificial mRNA bearing a perfect target site (Umbachet al., 2010; Bortoluzzi et al., 2011). Beyond this proof of principle experiment, a recent study reported moRNA specific expression in human embryonic stem cells (Asikainen et al., 2015) (hESCs). In the same study, moRNA and miRNA transfection experiments and microarray quantification of gene expression were conducted and identified gene silenced by moR-103a-2-3p, one of the most abundantly expressed moRNAs in hESCs, and by miR-103a. In line with these previous

studies, it was assumed that 3' -moR-128-2 can act as a miRNA, and investigated its possible impact on target gene silencing and on specific pathways or biological processes. To this end, it was considered how sequence variants (isomiRs) of these two small RNAs relate to each other. For miR-128-3p, it was identified 7 variants expressed in the analyzed $CD34^+$ samples: one exact isomiR, corresponding to the miRBase annotated mature form, and 6 " shorter or longer" variants (miR-128-3p-SL-1 to miR-128-3p-SL-6), whereas only 2 3'-moR-128-2 isomoR were found out (3'-moR-128-2-1 and 3'-moR-128-2-2). According to the conservative hypothesis that interprets moRNA as byproducts of Drosha cleavage (Ma et al., 2013) one should expect comparable mean levels of miRNA and moRNA cognate variants (i.e. obtained from a single endonucleolytic cleavage cut) in PMF and CTR samples. The observation that 3'-moR-128-2-1, the most abundant isomoR, is expressed only in CTR samples and its only viable cognate partner is the miR-128-3p-exact variant that is highly and nearly equally expressed in CTR and PMF samples does not apparently support such statements. Neither do the poor correlation of expression levels, in CTR and PMF samples, of cognate isomiRs and isomoRs, the fact that some abundant isomiRs are not associated to detected isomoR sequences, the good conservation of moRNA sequence (Fig 33C), and the previously reported observation that both isomoRs are not contained in the canonical hairpin (Figs 33A and 33B). Finally, target predictions of miRnas and moRnas resulted differentially expressed (miR-10b-5p, miR-19b-3p, miR-29a-3p, miR-379-5p, miR-543 and moR-128-2) were performed by using two different programs, miRanda (Enright et al., 2004) and PITA (Kertesz et al., 2007) which implement orthogonal target prediction strategies, and for which the code availability allowed us to make custom predictions of possible miRNA and moRNA target genes, by using as query sequences the identified isomiR and isomoR sequences. Among different isomiRs detected for each considered miRNA, it was considered the most expressed, even if it was different from the annotated sequence. It was also considered those isomiRs that were significantly contributing to miRNA total expression, and which were differently expressed in patients respect to controls (t-test p-value < 0.05 and $|\log2\ FC| > 1$), according to RNA-seq data. Accordingly, both isomoRs were considered for moR-128-2. After the analysis it was found that different miRNAs target genes converge to the same enriched pathways, as " Signaling in FGFR in disease", "DAP12 signaling" , "Oncogene induced senescence" and " Post-transcriptional silencing by small RNAs" .

Human fibroblast grow factor receptors (**FGFRs**) are a family of four tyrosine kinase receptors (FGFR1– 4), which are involved, in a variety of cellular processes. They are indeed key regulators of fibrogenesis, embryogenesis, angiogenesis, metabolism, and many other processes of proliferation and differentiation (Broocks et al., 2012; Tiong et al., 2013). Deregulation of FGFR signaling has been observed in numerous tumors (Katoh et al., 2014; Tan et al., 2014).

DAP12 is an immunoreceptor tyrosine-based activation motif (ITAM)-bearing transmembrane adapter molecule and it is reported to be signaling partner of activating natural killer receptors. DAP12 complex to TREM-1 and MDL-1 receptors to form receptor complexes involved in macrophage differentiation (Aoki et al., 2000)

and apoptosis in M1 leukemia cells (Aoki et al., 2002), significant monocytic activation of myeloid cell, calcium mobilization and inflammatory response (Gingras et al., 2002; Bakker et al., 1999). Its elevated expression levels are associates with enhanced cytotoxic characteristics in large granular lymphocyte leukemia (Chen et al., 2009).

Oncogene senescence (OIS) occurs when the activation of an oncogene is triggered; in this case it is termed oncogene-induced senescence. OIS acts as a barrier against tumour progression by driving stable growth arrest of cancer progenitor cells (Hills et al., 2014).

As anticipated, the putative targets of 3'-moR-128-2 and miR-128-3p were comoaratively analzyed. It was predicted the targets of the four most expressed miR-128-3p isomiRs (miR-128-3p-exact, miR-128-3p-SL-2, miR-128-3p-SL-3, and miR-128-3p-SL-4) and targets of the two 3'-moR-128-2 isomoRs (3'-moR-128-2-1 and 3'-moR-128-2-2), assuming that they would act as miRNA, as indicated by the available experimental data (Bortoluzzi et al., 2011). It was compared the union of predicted targets of miR-128-3p variants and the union of predicted targets of 3-moR-128-2 variants, to understand if and how much the moRNA function can be related to that of the cognate miRNA, as previously supposed (Asikainen et al., 2015). **Only a small fraction of 3-moR-128-2 target genes, less than 17%, is putatively targeted also by at least one of the miR-128-3p isomiRs.** Different pathways are enriched in predicted targets of 3' -moR-128-2 and of miR-128-3p. miR-128-3p targets are enriched in genes that are part of cellular pathways for the most part related to NGF, FGFR, ERBB4, ERBB2 signaling and transduction and to calcium ion homeostasis and signal transduction. Targets of 3' -moR-128-2 are enriched too in genes part of several, distinct, pathways related to cellular signaling in growth and proliferation as " Signaling by Notch", "Signaling by ERBB4", " Signaling by FGFR in disease" but also, quite interestingly, in genes part of the " Post transcriptional silencing by small RNAs" and of the more general "Regulatory RNA pathways". Remembering that 3'-moR-128-2 is highly expressed in normal and not detected in PMF. $CD34^+$, **it is worth notice that 4 out of 7 genes of the " Post-transcriptional silencing by small RNAs" path, namely AGO1, AGO3, TNRC6A, and TNRC6B, can be targeted by at least one isomoR of 3-moR-128-2.** Moreover, both considered 3-moR-128-2 isomoRs could also target RAN, the RAS-related nuclear protein, member of the RAS Oncogene Family, which is required for RNA export from the nucleus. In principle 3'-moR-128-2, where it is expressed, as in $CD34^+$ hematopoietic stem cells, could affect the expression of genes important for the entire process of miRNA-based silencing. It can indeed target genes essential for post-transcriptional silencing both by translation repression, as **AGO1/3**, and by mRNA degradation, as **TNRC6A/B**. **AGO1** and **AGO3** are required for post-transcriptional translation repression activity; **AGO1** is also involved in transcriptional silencing of promoters (Romero-Cordoba et al.,2014), and **AGO3** is also putatively involved into the modulation of mature miRNA incorporation to the RISC complex, thus controlling the ratio between micro- RNA guide and passenger strand (Winter et al., 2013). **TNRC6A**, and **TNRC6B** play a role in miRNA-dependent translation

repression and endonucleolytic cleavage, by recruiting specific deadenylase complexes.

The multifunctional protein RAN is involved in many processes and diseases: it controls cell cycle progression and it is a potential therapeutic target for treatment of cancers with activation of the PI3K/Akt/mTORC1 and Ras/MEK/ERK pathways (Yuen et al., 2012). Specifically in relation to the above mentioned findings, as known, RAN play a key role in RNA export from the nucleus and for the biogenesis of all miRNAs. Thus, RAN silencing by 3'-moR-128-2 can impair premiRNA transportation to the cytoplasm and output a reduction of miRNA biogenesis. A similar situation was documented by a recent study that identified, in Bombyx mori, a virus-encoded miRNA that suppresses the host miRNA biogenesis, exactly by targeting the host exportin-5 RAN cofactor (Singh et al., 2012).

The interest related to the study of the possible alteration in expression levels of genes and miRNA in PMF led to **extensively investigate also the expression of circulating miRNAs in plasma of PMF** patiens and healty donors in order to identify new potential biomarkers for this disease. This study was encouraged by the fact that many recent works demonstrated that the deregulated expression level of circulating miRnas can be biomarker of various form of cancer (see Par 2.4.7).

In this part of study, **6 differentially expressed miRNA resulted deregulated in significant statistically way (P value<0.05): miR-let7b*, miR-10b-5p, miR-424 and miR-99a were resulted up-regulated instead miR-144* and miR-375 were down-regulated in PMF patients compared to the controls.** Between these miR-99a and miR-144* were resulted already equally de-regulated in granulocytes of PMF patients in a previous work (Guglielmelli et al., 2007).

Also this time, the **miR-10b-5p is deregulated but in contrast with the data obtained in $CD34^+$ and granulocyte of PMF in which it was down-regulated.** In addition, very interesting was the down-regulation of **miR-375**.

MiR-375 expression was decreased in most human cancer and overexpression of miR-375 suppressed cancer cell proliferation and invasion, indicating that miR-375 might be a tumor suppressor (Fig. 34).

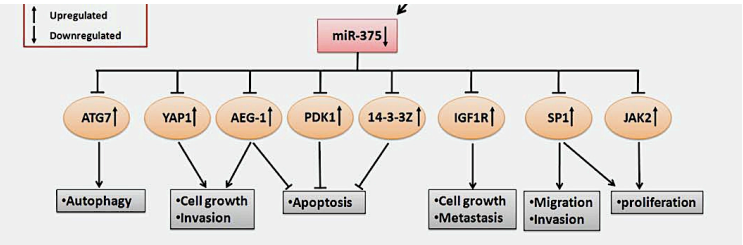


Fig.34: Role of tumor suppressor and main targets of miR-375.

The dysregulation of **miR-375** expression in different human cancer is caused by a variety of mechanisms, such as the dysregulation of transcription factors, aberrant promoter methylation, and aberrant histone deacetylation. Promoter DNA hypermethylation leading to the downregulation of miR-375 was frequently occurred

in hepatocellular carcinoma (Furuta et al., 2010), oesophageal squamous cell carcinoma (ESCC) (Kong et al., 2012), and other cancers (Chu et al., 2014).

Recently, Xu et al. indicated that the down-regulation of miR-375 in plasma is matched in colorectal cancer (Xu et al., 2014) and Yin et al. already demonstrated the importance of miR-375 as a tumor suppressor in MPN by targeting JAK. They found **miR-375** expression is increased in JAK2V617F-positive patients compared with JAK2V617F-negative patients. **This negative correlation indicates MPN patients with low expression of miR-375 may have high expression of JAK2**, which might contribute to the further activation of JAK2/STAT signaling. Conseguently, Yin et al. proposed the combination of the JAK2 kinase inhibition and restoration of miR-375 expression as a possible strategy for clinical therapies of MPN (Yin et al., 2015). On the basis of our and litterature data, it is possible to conclude that **miR-375 can be a good candidate as a biomarker of disease in PMF patients plasma.**

Finally, in order to clarify also the contribution of miRNAs to the pathogenesis of JAK2V617F-positive MPNs, it was analysed the miRNAs expression pattern in erythroid (TER119+) and mieloid ($GR1^+$) cells purified from BM of JAK2V617F knock-in (KI) mouse model. At the end of this work, it was confirmed up-regulation of miR-7a-5p and miR-291b-5p ad down-regulation of miR-150-5p in GR1+; in TER119+, up-regulation of miR-7a-5p and down-regulation of miR-150-5p and miR-592-5p. Only 2 differentially expressed miRNAs were shared by both erythroid and myeloid cells (miR-7a-5p and miR-150-5p, up- and down-regulated respectively), suggesting a direct relationships between these miRNAs and JAK2V617F. Infact, to confirm this relationship, Gebauer et al. already demostrated that the expression of miR-150 could further be shown to correlate with JAK2 allele burden in MPNs (Gebauer et al., 2013). Indeed, Bruchova et al. showed that the expression of miR-150 progressively declines with eritroid differentiation and the down regulation, in particular in PV, suggested possible involvement of miR-150 in pathogenesis of this disease (Bruchova et al., 2007). miR-150 is predicted to target cMYB proto-oncogene, therefore, a significant decrease of miR-150 and a simultaneous increase of the expression level of this gene may be responsible for maintaining proliferation at early stages and for the inhibition of terminal erytroid differentiation.

7 FINAL STATEMENTS

At the conclusion of this work, I can summarize the main points as follows: 1- novel data regarding the expression profile of small RNA in $CD34^{+}$, granulocytes and plasma of PMF patients as well as of healthy controls and their possible impact on specific genes and pathways have been provided; 2- new, previously undescribed moRNAs have been reported which might contribute to disease pathogenesis; 3- a number of differentially expressed miRNAs in *JAK2V617F* KI mouse have been identified whose deregulation very likely contributes to the development/phenotype of MPN in this unique model. Overall, these information may represent the basis for further studies aimed at a deeper knowledge of the prognostic and therapeutical role of miRNAs and also moRNAs in PMF.

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